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**13. ABSTRACT** *(Maximum 200 words)*

Corticotropin Releasing Hormone, CRH, is a 41 amino acid peptide expressed primarily in the parvocellular neurons of the paraventricular nucleus of the hypothalamus. CRH regulates the synthesis and secretion of glucocorticoids which are the end product of the hypothalamic-pituitary-adrenal (HPA) axis. The CRH gene and peptide are conserved across numerous animal species indicating the importance of CRH in regulating the HPA axis. In addition to expression in the hypothalamus, CRH is expressed in various peripheral tissues including the placenta but expression in this tissue is uniquely species-specific. Only humans and high primates express the gene in their placentas, indicating that unique mechanisms, distinct from those controlling expression in the hypothalamus, have evolved to control expression in placenta. The goal of my studies has been to elucidate these mechanisms, using choriocarcinoma cell lines as a model for placental trophoblasts. The results from my studies indicate that differences in cellular *trans*-acting factors rather than in *cis*-acting sequences dictate the species-specific placental expression of CRH. Three species-specific candidate nuclear factors have been identified which may contribute to the placental expression of CRH. These factors include a 58-kDA human specific activator capable of binding to a cAMP responsive region located at -200 to -99 bp in CRH.

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## Table of Contents

Corticotropin Releasing Hormone, CRH, is a 41 amino acid peptide expressed primarily in the parvocellular neurons of the paraventricular nucleus of the hypothalamus. CRH regulates the synthesis and secretion of glucocorticoids which are the end product of the hypothalamic-pituitary-adrenal (HPA) axis, and are essential for the daily survival of an organism. The CRH gene and the peptide are conserved across numerous animal species indicating the importance of CRH in regulating the HPA axis.

In addition to its expression in the hypothalamus, CRH is expressed in the placentas of humans and higher primates but not in the placentas of rodents or lower primates. The site of transcription initiation for CRH mRNA and the CRH peptide sequence are identical in both brain and placenta, indicating that some mechanisms controlling expression in these tissues may overlap. However, the species-specific expression pattern of CRH in the placenta implies that unique mechanisms distinct from those controlling expression in hypothalamus regulate expression in this tissue. The unique properties of CRH expression in the placenta make it an interesting gene to investigate the mechanisms controlling tissue specific gene expression. By comparing the patterns of expression of the human and mouse genes in tissue culture cells and in transgenic mice, I have tested the hypothesis that the presence of specific *cis*-acting sequences dictate the patterns of CRH gene expression in different animal species.

Using BeWo and JEG-3 choriocarcinoma cell lines as models for human trophoblasts, transient transfection experiments demonstrate regulated expression of human CRH (hCRH)-luciferase reporter genes. In comparison, little to no expression is detected in either a non-specific monkey kidney cell line, CV-1, or in the rodent choriocarcinoma cell line Rcho-1 which serves as a model for rodent trophoblasts. When a mouse CRH-luciferase reporter gene is transfected into the human and rodent choriocarcinoma cell lines, it behaves similarly to the corresponding hCRH reporter gene. It is expressed in the human BeWo cells but has low levels of expression in the rodent Rcho-1 cells.

Deletions of the human CRH promoter identify control regions that contribute to the species-specific expression pattern of CRH in the placenta. Three regions have been identified that contribute to the species-specific expression of CRH in placenta and candidate

nuclear factors from either human or rodent cell lines have been identified that bind to these regions. These studies, using human and rodent choriocarcinoma cell lines as models of placental trophoblasts, demonstrate that differences in cellular *trans*-acting factors rather than in *cis*-acting sequences dictate the species-specific placental expression of CRH. **(See attached manuscript: Scatena CD and Adler S. 1996. *Trans*-Acting Factors Dictate the Species-Specific Placental Expression of Corticotropin-Releasing Factor Genes in Choriocarcinoma Cell Lines. *Endocrinology* 137: 3000-3008.)**

The transcriptional response to cAMP contributes to the specific expression of CRH. A major part, but not all, of this effect is mediated by the canonical cAMP response element (CRE) conserved in mouse, rat, and human CRH promoters. In addition to the CRE at -220 base pair (bp) in hCRH, fine mapping studies have identified a 20 base pair cAMP responsive region located at -128 to -109 bp within the hCRH promoter. I have identified, in human but not in rodent trophoblasts, a 58 kDA DNA binding protein which binds to this 20 bp site. Transfection studies indicate that the 58 kDA protein alters the cAMP responsiveness of this region. This human-specific factor contributes to the species-specific expression of CRH in human trophoblasts. **(See attached manuscript: Scatena CD and Adler S. 1998. Characterization of a Human Specific Regulator of Placental Corticotropin Releasing Hormone. *Molecular Endocrinology* 12: 1228-1240.)**

In addition to the transfection experiments in the human and rodent cell lines, transgenic mice have been created, carrying a transgene composed of 5 kb of the hCRH promoter linked to the coding region of neomycin phosphotransferase II (NEO), to examine the expression of hCRH in mouse placenta. Evaluation of four independent lines of transgenic mice confirm targeted and regulated expression of the transgene in the hypothalamus. The expression of the transgene in other organs, in both males and females, is similar to the expression pattern of the endogenous mouse CRH gene. Evaluation of transgenic placentas, however, shows no expression of the hCRH transgene in 21 of 22 specimens. Therefore, hCRH transgene expression in placenta is similar to the endogenous mouse CRH gene, rather than reflecting the human expression pattern. The lack of placental expression of the hCRH transgene supports my cell culture studies which indicate that

species-specific *trans*-acting factors play a dominant role in determining the placental expression of the hCRH gene. **(See attached manuscript: Scatena CD, Ramkumar TP, and Adler S. Expression of Human CRF Transgenes in Transgenic Mice: Analysis of Species-Specific Placental Expression.)**

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# *Trans*-Acting Factors Dictate the Species-Specific Placental Expression of Corticotropin-Releasing Factor Genes in Choriocarcinoma Cell Lines*

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**ABSTRACT**

CRF, in addition to its role in the hypothalamus, demonstrates species-specific expression in the placentas of higher primates, but not rodents. Transient transfections of BeWo and JEG-3 choriocarcinoma cells, as models for human trophoblasts, demonstrate regulated expression of human (h) CRF-luciferase reporter genes, whereas little or no expression is detected in other lines, including CV-1 cells. The rodent choriocarcinoma cell line, Rcho-1, a model for rodent trophoblasts, is defective in the expression of transfected hCRF genes. The mouse CRF promoter behaves similarly to the corresponding hCRF construct. It is active in BeWo and inactive in Rcho-1 cells.

The transcriptional response to cAMP contributes to the specific expression of CRF. Analyses of deleted or mutated hCRF promoters identify a key role for protein kinase A-dependent pathways. A major part, but not all, of this effect is mediated by the canonical cAMP response element conserved in mouse, rat, and human CRF promoters. Additional deletions of the human CRF promoter identify control regions that also contribute to the observed species-specific expression pattern, and each identified region binds factors in nuclear extracts derived from the appropriate cell line. These studies using human and rodent choriocarcinoma cell lines as models of placental trophoblasts demonstrate dominant effects of cellular *trans*-acting factors, rather than DNA sequence differences, in dictating the species-specific placental expression of CRF. (*Endocrinology* **137:** 3000–3008, 1996)

THE HYPOTHALAMIC peptide CRF plays a key role in regulating the hypothalamic-pituitary-adrenal axis. CRF production is a critical first step in the synthesis of glucocorticoids, which are essential for life and an integral component of mammalian carbohydrate metabolism, and the stress response. The peptide sequence and expression of CRF are conserved across numerous animal species, indicating their importance in the maintenance of mammalian homeostasis (1). In addition to the hypothalamus, CRF is expressed in various peripheral tissues, including the placenta (1); however, its expression in this organ is uniquely species specific (2). The placentas of humans and higher primates express CRF messenger RNA (mRNA), whereas those of the rat, mouse, lemur, and guinea pig fail to express the gene (2, 3).

Recent studies indicate that CRF and its specific binding protein (4) act as a clock to time the onset of human labor (5). However, the precise role of placental CRF and its binding protein in the human physiology of fetal development and parturition has yet to be determined. From the seventh week of gestation until parturition, CRF mRNA is detected in human placenta (6). There is a gradual increase in expression of the gene during the course of a pregnancy; it increases dramatically during the last 5 weeks before delivery (6). Studies indicate that placental CRF may increase the production of PGs, known mediators of labor, and it may also potentiate the effect of oxytocin on uterine contractions (7). CRF produced in the placenta may enter the fetus and stimulate the fetal pituitary-adrenal axis, resulting in the increase in cortisol seen in fetal plasma during the last 5 weeks of pregnancy (8). The cortisol surge may allow proper maturation of fetal organs and serve as one of the signals necessary for the initiation of labor (8, 9). Alternatively, placental CRF may act in a paracrine fashion, stimulating the release of ACTH from the placenta (8), thereby influencing the fetal adrenal glands (8). CRF, acting as a lymphokine, might also modulate the immune relationship between the fetus and the mother (10).

Expression of CRF in placenta represents a distinct system for investigation of both cell type- and species-specific gene expression as well as a means to gain insight into the function of placental CRF in human physiology. In this work we have exploited the availability of human and rodent choriocarcinoma cell lines as models of placental trophoblasts together with promoter sequences from both the human and mouse CRF genes to investigate the molecular basis of the human-specific placental expression of CRF. Unlike previous studies of other human-specific placental genes, our results indicate a dominant role for species-specific *trans*-acting factors, rather than DNA sequence differences, in determining the expression pattern of the CRF gene.

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## Materials and Methods

### *Luciferase plasmids*

Luciferase reporter plasmids are constructed in a specially modified vector, LΔ2S. The vector is derived from pBLCAT2 (11), modified by replacing the chloramphenicol acetyltransferase (CAT) gene with a luciferase reporter, by incorporating simian virus 40 (SV40) termination signals upstream of the promoter, and by removing potential activator sites from both the plasmid backbone and the luciferase-coding region. The activating protein-1-related sequence aTGTGTCAga, at nucleotides 1221–1232 of the luciferase gene (12), was replaced by aTGTGTGCga using site-directed mutagenesis (13), a change that does not result in changes in the amino acid sequence. An identical aTGTGTCAga sequence in the pBLCAT2 backbone was removed by digestion with *Dra*II and *Nde*I. After replacing the CAT-SV40 region with the mutated luciferase-SV40 sequence, triplet termination signals (14) were inserted upstream of the promoter. This plasmid contains 5′-*Bam*HI and 3′-*Xho*I sites for insertion of promoter sequence cassettes.

### *Human and mouse CRF promoters*

The human CRF genomic clone, SpHCRH-1, was the generous gift of Shosaku Numa (15). The 5-kilobase (kb) upstream region was isolated as a *Eco*RI-*Xho*I fragment after *Tth*III1 partial digestion (cutting at +13 bp), followed by filling and ligation to an *Xho*I linker. The 532-bp promoter was isolated by a complete *Tth*III1 digest. Additional deletions of the hCRF promoter were made by specific restriction digests or PCR. Deletions were confirmed by dideoxy sequencing.

The human (h) CRF-luciferase fusion construct contains the LΔ2S backbone and the 8-kb hCRF genomic clone. The mutated luciferase gene has been placed in-frame in the second exon, replacing the coding region of the hCRF prepropeptide. This construct contains 5 kb of 5′-promoter, the first exon and intron, the modified second exon encoding luciferase, and the 3′-flanking region.

The mouse CRF gene was isolated by screening a mouse genomic library using oligonucleotides contained in the 5′-proximal promoter and the second exon of the hCRF gene. After plaque purification of a single λ- mouse (m) CRF clone, the mCRF promoter was sequenced using the Promega PCR cycle sequencing kit (Promega Corp., Madison, WI). Oligonucleotides were designed to isolate the 536-bp promoter cassette by PCR. The 536-bp promoter was inserted into the LΔ2S luciferase vector.

### *Mutagenesis*

Oligonucleotide-directed mutagenesis was performed in phagemid vectors using minor modifications of the method of Kunkel (13). The cAMP response element at −220 bp in the hCRF promoter was changed from TGACGTCA to GGAATTCC.

### *Expression vectors*

Plasmids containing the Rous sarcoma virus (RSV) promoter for expressing the catalytic subunit of protein kinase A, Rous sarcoma virus-protein kinase A (RSV-PKA), and the heat-stable inhibitor of the cAMP-dependent protein kinase (PKI), RSV-PKI, were obtained from Richard Maurer (16, 17). The RSV-Neo plasmid, expressing the neomycin phosphotransferase II gene, was as previously described (18).

### *Cell lines*

HeLa, CV-1, MDA-MB-231 (MB), BeWo, JAr, and JEG-3 cell lines were obtained from the American Type Culture Collection (Rockville, MD). In addition, a subclone of BeWo cells, b30, was the gift of A. Schwartz (19). BeWo cells from both sources behaved similarly in these experiments. Rcho-1, rat choriocarcinoma cells, were the generous gift of M. Soares (20). HeLa, CV-1, and MDA-MB-231 cells were grown in 10% $CO_2$ in DMEM with 5% FBS and 5% enriched calf serum (ECS; Gemini Bioproducts, Calabasa, CA). BeWo and Rcho-1 cells were grown in 5% $CO_2$ in NCTC-135 with 5% FBS, 5% ECS, 0.4% glucose, 50 μM 2-mercaptoethanol, and 100 μM sodium pyruvate. JEG-3 cells were grown in 5% $CO_2$ in MEM with 5% FBS and 5% ECS. JAR cells were grown in 5% $CO_2$ in RPMI 1640 with 10% FBS. All of the above growth media were supplemented with antibiotics. All cells are routinely surveyed for mycoplasma using a PCR method from Stratagene (La Jolla, CA).

### *Transfections*

Transient transfections were performed using a calcium-phosphate method (21) in either 100-mm plates or 35-mm 6-well plates. Typically, for a 6-well plate, 100,000 cells/well were seeded in growth medium 2 days before transfection. On the day of transfection, cells were fed with DMEM containing 10% FBS and incubated in a 10% $CO_2$ environment. Four hours later, each 2-ml well was transfected with 150 μl *N,N*-bis (2-hydroxyethyl)-2-aminoethane-sulfonic acid (BES)-buffered saline (BBS)-$CaCl_2$ solution containing a total of 6 μg DNA. The DNA solution consisted of 3 μg luciferase reporter plasmid and salmon sperm DNA to bring the final DNA concentration to 6 μg. Alternatively, some experiments used 2.5 μg total DNA, with the same ratios of reporter and carrier DNA. Plates were then placed overnight in 5% $CO_2$. The next day, cells were rinsed with DMEM, fed their growth media, and hormone treated as indicated. One day after hormone treatment, cells were harvested in 150 μl of a Triton lysis buffer containing 50 mM Tris (hydroxymethyl)aminoethane, 50 mM 2(*N*-morpholino)ethane sulfonic acid (pH 7.8), 1 mM dithiothreitol, and 1% Triton X-100. The lysate was assayed for luciferase activity as previously described (22), using an Analytical Luminescence Laboratories (San Diego, CA) Monolight 2010 luminometer. β-Galactosidase assays were performed using chlorophenol red β-galactopyranoside (Boehringer Mannheim, Indianapolis, IN) as substrate (23) and an Anthos plate reader (Anthos Labtec Instruments, Salzburg, Austria) with Delta Soft II software (Bio Metallics, Princeton, NJ).

For transfection experiments, the data shown, when indicated, are luciferase values normalized to the basal activity of the herpes thymidine kinase promoter set at 100. Experiments determining the basal activity of CRF promoter constructs in BeWo and Rcho-1 cells showed indistinguishable results with or without inclusion of either pCH110 (Pharmacia, Piscataway, NJ) or an RSV β-galactosidase reporter and internal standardization. These β-galactosidase reporters are not neutral to hormone treatment, as we observed effects of forskolin, 8-bromo-cAMP (8-Br-cAMP), and PKA expression on β-galactosidase activity. In addition, we observed promoter interference that varied with the inherent strength or stimulated activity of each tested CRF luciferase deletion construct. For these reasons, all data presented were obtained from experiments without inclusion of additional β-galactosidase reporter plasmids for internal standardization.

### *Electromobility shift assays*

Cell extracts from cultured cells were prepared using minor modifications of a microtechnique (24). DNA fragments were prepared by PCR and purified using PAGE. Probes were labeled using direct incorporation of radioactive nucleotides during PCR or with T4 polynucleotide kinase. For the human-specific activator, binding reactions contained 5–20 μg nuclear extract, binding buffer (25), 5 mM $MgCl_2$, 50 mM $NaPO_4$ (pH 7), and 5 μg poly(dI·dC)·poly(dI·dC). Cold competitor, when included, was at an approximately 100-fold molar excess. The final binding reaction volume, including probe, was 20 μl. Binding reactions were preincubated at room temperature for 10 min before the addition of probe. After probe addition, reactions were incubated overnight at 0 C to achieve binding equilibrium. Polyacrylamide gels (4% acrylamide-bis, 38:2) were electrophoresed at 4 C at 10 mA. Gels contained 2.5% glycerol and 0.5 × glycerol-tolerant gel buffer (Tris-Taurine-EDTA, TTE) (U.S. Biochemical Corp., Cleveland, OH). For the rodent activator, the binding reaction contained 5 or 10 μg nuclear extract. For the rodent repressor, the binding reactions contained 5 μg nuclear extract. Each reaction also contained 5 μg poly(dI-dC) and binding buffer containing 12% glycerol, 12 mM HEPES (pH 7.5), 50 mM KCl, 1 mM $MgCl_2$, 20 μg/ml BSA, and 5 mM dithiothreitol. Cold competitor, when included, was at approximately a 100-fold molar excess. The final binding reaction volume, including probe, was 20 μl. Binding reactions were preincubated at room temperature for 10 min before the addition of probe. After probe addition, reactions were incubated for an additional 20 min before elec-

trophoresis. Polyacrylamide gels (4% acrylamide-bis, 80:1) were electrophoresed at 4 C at 1000 V. Gels contained 2.5% glycerol and 0.5 × TTE. Results were visualized using autoradiography at −80 C with an intensifying screen or by storage screen analysis.

## Results

*Human choriocarcinoma cell lines specifically express hCRF reporter genes*

Choriocarcinoma cell lines have been widely used as a model of placental trophoblasts for studies of gene expression. These cell lines have been used in the analysis of placental gene expression, including CGα (19, 26–30) and been shown to contain *trans*-acting factors necessary for tissue-specific expression of this gene (26–29). We determined whether choriocarcinoma cells were an appropriate model for studying the expression of human CRF by analyzing these cells for expression and regulation of transfected hCRF luciferase reporter genes.

Two human choriocarcinoma cell lines, BeWo and JEG-3, were chosen as models for human placental trophoblasts. The cells were transfected with either of two luciferase reporter gene constructs (Fig. 1A). The first construct contains 5 kb of 5′-proximal hCRF sequence linked to the firefly luciferase reporter gene. The second construct uses the 8-kb hCRF genomic clone (15) in which the sequences encoding the CRF peptide were removed and replaced in-frame with the sequences encoding firefly luciferase (12). The construct retains the first exon, the intron, part of the second exon, the 3′-untranslated region, and the polyadenylation site from the human genomic clone. In transient transfection experiments, the expression of both constructs varied in different cell lines (Fig. 1B). In the human choriocarcinoma cell lines as well as the nonplacental CV-1 cells, basal levels of reporter gene expression were low (Fig. 1B). Upon stimulation of the PKA pathway with forskolin, the pattern of expression in the two cell types changed dramatically. Treatment of the human choriocarcinoma cell lines with forskolin resulted in an increase in CRF gene expression of approximately 8-fold, which did not occur in the nonplacental cell lines (Fig. 1B). Similar noninducible expression patterns were observed for the human HeLa and MB-231N nonplacental cells (data not shown), whereas a similar inducible pattern was observed for the human JAr choriocarcinoma cell line (data not shown).

The genomic fusion reporter gene displays lower basal levels of luciferase activity than the 5-kb promoter in CV-1 cells. This suggests that sequences outside the 5′-flanking region contribute to tissue-specific expression, in agreement with previous observations (31). However, the human choriocarcinoma-specific expression of the hCRF reporter gene

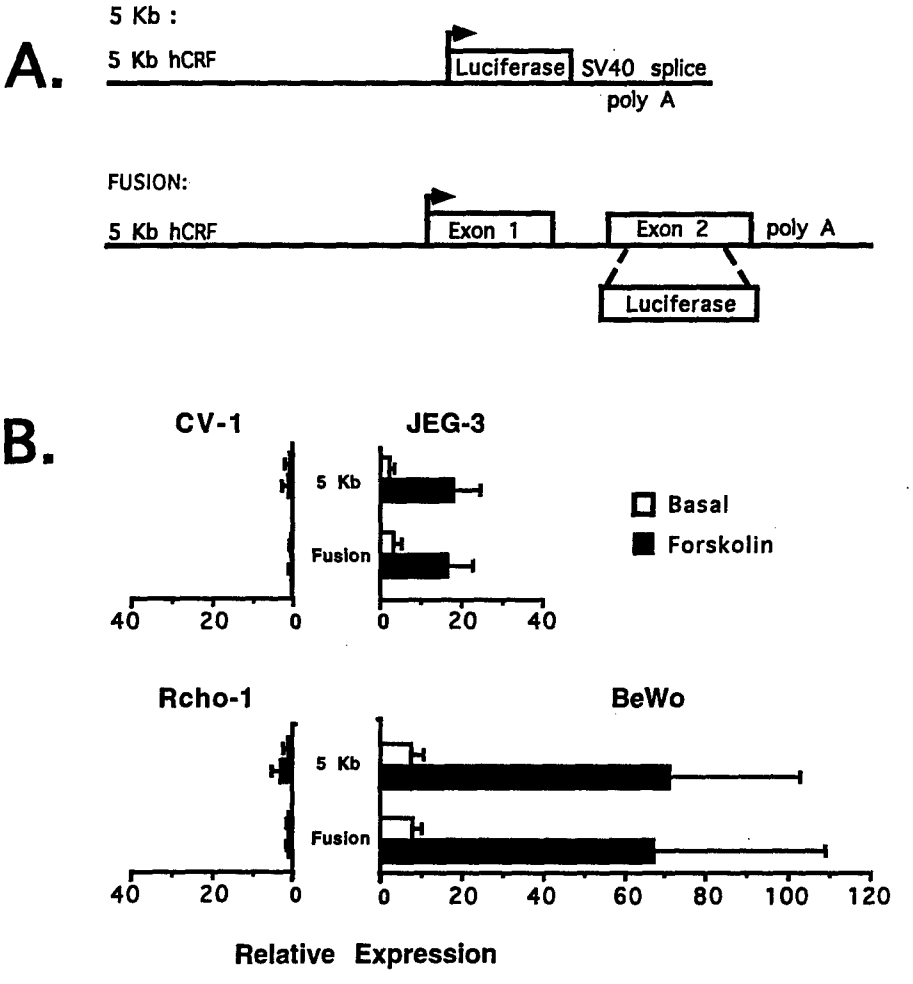


FIG. 1. A, hCRF luciferase reporter genes. The 5-kb CRF construct contains the 5′-flanking sequence of CRF fused to firefly luciferase, followed by the SV40 splice and polyadenlyation signal sequences. CRF-Fusion consists of the 8-kb hCRF genomic clone with the coding region for the CRF peptide removed and replaced with the sequence encoding firefly luciferase as an in-frame fusion. The *arrow* indicates the transcription start site. B, Cell type-specific expression of hCRF. JEG-3 and BeWo are human choriocarcinoma cell lines. CV-1 is a monkey kidney fibroblast line. Rcho-1 cells are a rat choriocarcinoma cell line. Using a standard calcium phosphate protocol (see *Materials and Methods*), the cells were transfected with the indicated reporter genes. Twenty-four hours posttransfection, cells received either vehicle or 25 μM forskolin; 24 h posthormone treatment, cells were harvested for luciferase assay (see *Materials and Methods*). Relative expression indicates luciferase values normalized to herpes thymidine kinase (TK) equal to 100. Results are the mean ± SEM from three experiments.

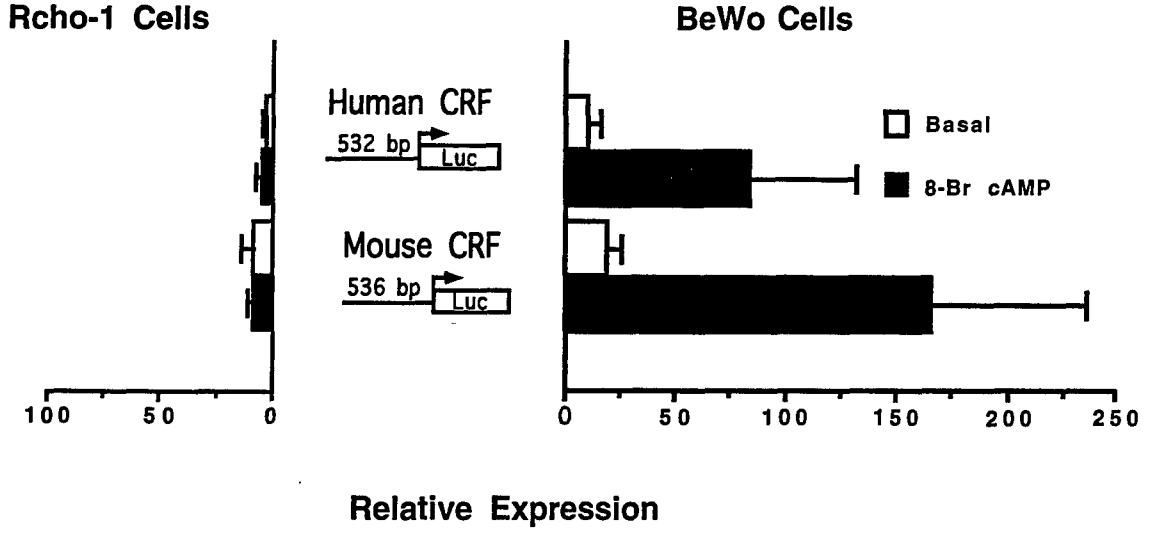


FIG. 2. Expression of human *vs.* mouse CRF in human and rodent choriocarcinoma cell lines. Human BeWo and rodent Rcho-1 cells were transfected with the indicated human or mouse CRF reporter genes. Twenty-four hours posttransfection, the indicated cells received 1 mM 8-Br-cAMP; 24 h posthormone treatment, cells were harvested for luciferase assay (see *Materials and Methods*). Relative expression indicates luciferase values normalized to herpes thymidine kinase (TK) equal to 100. Results are the mean ± SEM from three experiments.

containing only the 5′-flanking region indicates that *cis*-acting sequences within this region must also contribute to the observed tissue-specific expression pattern. Thus, the 5′-proximal promoter can be used in experiments to determine *cis*-acting sequences important for the species-specific expression of CRF in these cell lines. These results demonstrate that human choriocarcinoma cell lines are a suitable model for studying CRF expression and contain factors necessary for the regulated and specific expression of human CRF reporter genes.

*Activity of the Rcho-1 cell line*

The Rcho-1 cell line has been used as a model for previous studies of rodent trophoblast gene expression, including mouse placental lactogens I and II and P450SCC (20, 32, 33). We, therefore, performed a series of transient transfection experiments with CRF reporters using this cell line as a model for rodent placenta. There was little or no expression of the hCRF reporter genes in these cells, even after treatment for 24 h with forskolin (Fig. 1B). Because the same reporter constructs were effectively expressed in the human choriocarcinoma cell lines, the results suggest that *trans*-acting factors contribute to the lack of expression of CRF in this rodent choriocarcinoma cell model.

*Expression of the mCRF gene in human and rodent cell lines*

In addition to differing *trans*-acting factors that might control human and rodent CRF expression, DNA sequence differences might also contribute to this species-specific expression pattern. To determine whether DNA sequences contained in the mouse CRF gene might restrict its expression, a genomic clone of mouse CRF was isolated. Reporter gene constructs were made using 532 bp of the hCRF promoter and a corresponding 536-bp mouse CRF promoter. The two constructs were used in parallel in both BeWo and

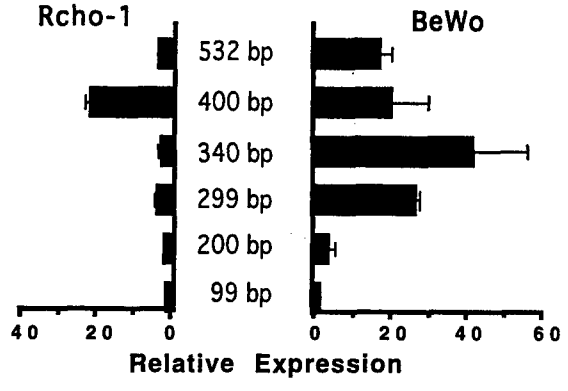


FIG. 3. Comparison of the basal expression of hCRF in human and rodent choriocarcinoma cell lines. Human BeWo and rodent Rcho-1 cells were transfected with the indicated reporters and harvested for luciferase assays (see *Materials and Methods*). Relative expression indicates luciferase values normalized to herpes thymidine kinase (TK) equal to 100. Results are the mean ± SEM of two experiments.

Rcho-1 cells. As shown in Fig. 2, in BeWo cells, both the human and mouse reporters were expressed and induced by the addition of 8-Br-cAMP. In contrast, in the Rcho-1 cell line, both constructs had lower basal expression and were not responsive to 8-Br-cAMP induction.

These results suggest that it is cellular factors differing between human and rodent choriocarcinoma cells rather than sequence differences between human and rodent CRF promoters that dictate the observed species-specific CRF expression. The results also imply that there are common *cis*-acting sequences that control CRF expression within the first 536 bp of mouse and human CRF 5′-flanking sequences.

*Deletional analysis of the CRF promoter*

To more precisely identify *cis*-acting sequences that participate in species- and cell type-specific CRF expression,

deletions of the human promoter were analyzed for expression. A series of chimeric luciferase reporter plasmids containing deletions of the human CRF 5'-proximal region was created using restriction enzyme digestion and PCR. Basal activities of this deletion series were determined in parallel in BeWo and Rcho-1 cells (Fig. 3). The different activities of each promoter in the human and rodent cell lines again show that *trans*-acting factors differing between human and rodent cells affect the species-specific expression of CRF (Fig. 3). Unlike the 532-bp promoter, which has low activity in Rcho-1 cells, the 400-bp CRF promoter displays higher basal expression in the rodent cells, essentially equivalent to the activity in human BeWo cells (Fig. 3). This suggests that factors interacting with the 132-bp segment from −532 to −400 limit the expression of the CRF promoter in the rodent cells. The 340-bp promoter shows similar elevated basal expression in BeWo cells, but decreased expression in the Rcho-1 cell line (Fig. 3). Factors interacting with the 60-bp sequence from −400 to −340 thus serve to activate expression of the hCRF gene in the rodent cell line. Further deletions of the hCRF promoter all displayed weak activity in Rcho-1 cells. In contrast, basal expression in BeWo cells remained elevated until removal of the region from −299 to −200. This region contains a canonical CRE. Although this 100-bp region may contain other important regulatory sites, these results suggest the potential importance of the CRE element in controlling basal as well as activated expression of CRF in human BeWo cells and indicate that the PKA pathway may be partially activated even under basal conditions.

*Analysis of the PKA pathway*

In an effort to determine whether the CRE is involved in regulating CRF gene expression, hCRF-luciferase constructs were created in which the canonical CRE element was mutated. The 5-kb X-CRE and 532-bp X-CRE luciferase constructs were transfected into BeWo cells (Fig. 4). The mutation of the CRE element resulted in a decrease in basal expression in BeWo cells (Fig. 4, *top*). Unexpectedly, although the CRE site was mutated, the X-CRE constructs still retained a 4-fold induction with 8-Br-cAMP (Fig. 4, *bottom*). This effect was seen not only in the 5-kb promoter, but also in the 532-bp

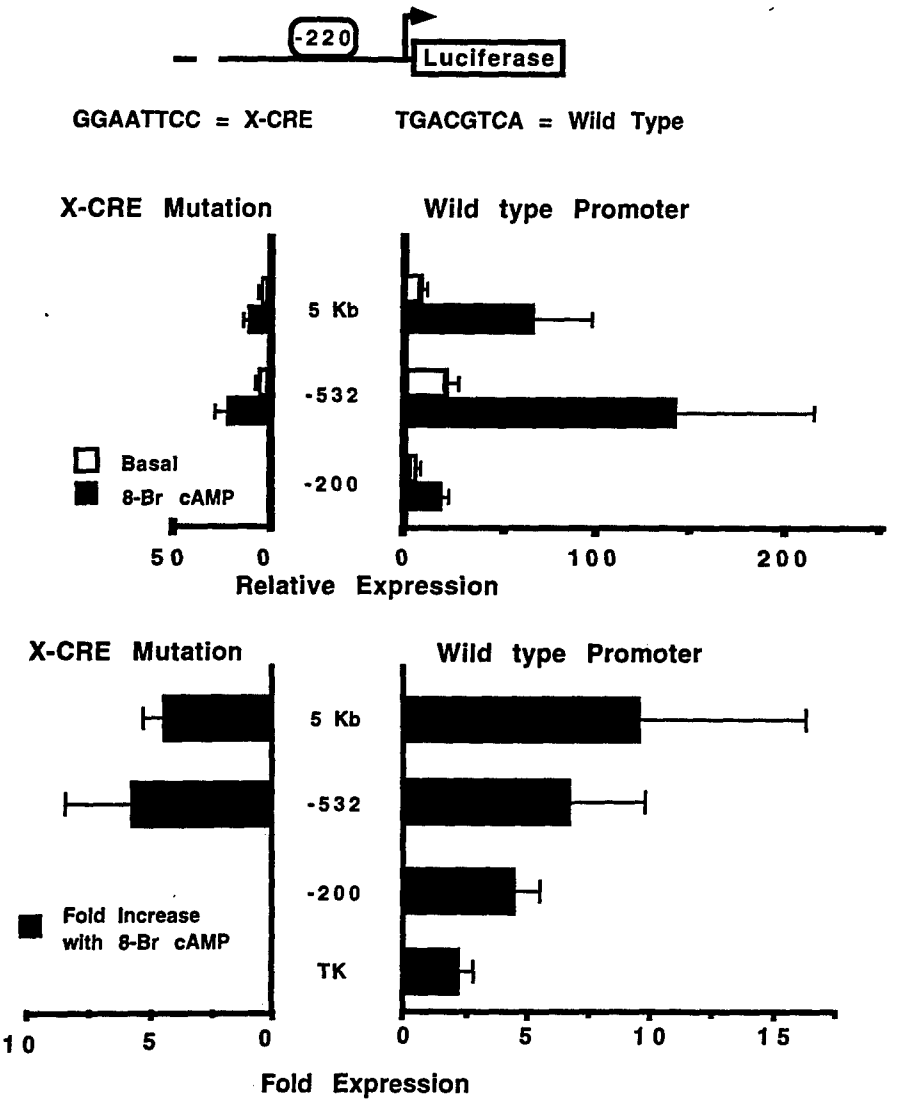


FIG. 4. Effect of the CRE element on CRF expression. X-CRE constructs containing the sequence GGAATTCC, which replaces the canonical CRE at −220, were created using site-directed mutagenesis. BeWo cells were transfected with wild-type or mutated promoters as indicated. Cells were treated with 1 mM 8-Br-cAMP 24 h after transfection and harvested after an additional 24 h. *Top*, Relative expression indicates luciferase values normalized to herpes thymidine kinase (TK) equal to 100. *Bottom*, Fold indicates the relative increase in expression with hormone treatment for each promoter construct. Data are the mean ± SEM from three experiments.

promoter and even in the 200-bp CRF promoter, which lacks the canonical CRE sequence (located at −220). In contrast, only a 2-fold induction was seen with the control thymidine kinase promoter (Fig. 4, *bottom*). Comparison of the fold activation of the 5-kb, −532, and −200 promoters (Fig. 4, *bottom*) as well as additional deletion constructs of the hCRF promoter with or without a mutated CRE (data not shown) suggests that several promoter regions may contribute to this CRE-like effect. This effect occurs in human BeWo cells, but is not observed in Rcho-1 cells (data not shown) and, thus, is species specific. Similar preservation of cAMP responsiveness was observed with forskolin treatment or direct activation by cotransfecting the PKA catalytic β-subunit (17) (data not shown). These results indicate that either there is a variant CRE element not yet identified within the −200 5′-flanking sequence, or there is another *trans*-acting factor influenced by the PKA pathway that interacts with these regions. The cAMP regulatory pathway is known to be very important in controlling the expression of placental genes (34). It appears that this regulatory pathway is involved in the control of the CRF gene in human BeWo cells.

*Species-specific trophoblast factors bind to the hCRF promoter*

The experiments presented above suggest that trophoblast *trans*-acting factors, rather than DNA sequence differences, dictate the species-specific expression of CRF. Additionally, deletional analysis of the human promoter has identified potential regulatory sites. To study this further, we performed gel shift assays with nuclear extracts from human JEG-3 and BeWo cells and rodent Rcho-1 cells, using the DNA promoter sequences that contribute to species-specific expression identified by transfectional analyses. Figure 5 shows the results of a comparison of nuclear extracts binding to a 82-bp fragment of the hCRF gene from −150 to −68 that contains sequences responsive to PKA stimulation in BeWo cells. A common, low band is seen in extracts from both human cell lines, JEG-3 and BeWo (Fig. 5, *left panel*), specifically competed by excess unlabeled fragment (Fig. 5, *right panel*). Notably, this band is reduced or absent in the Rcho-1 extract, although extracts bind this fragment with a different mobility (Fig. 5, *left panel*). These results further support the conclusion that *trans*-acting factors differing between rodent and human choriocarcinoma/trophoblast cells are responsible for dictating the species-specific pattern of placental CRF gene expression.

In addition to the human-specific PKA-responsive activator, two other regions of interest were identified by transfectional mapping. A repressor sequence was identified in Rcho-1 cells at −532 to −400 bp that when removed increases expression of the hCRF promoter. Gel shift analysis showed that factors present in Rcho-1 cells bound this region and were specifically competed by excess unlabeled fragment (Fig. 6, *left panel*). Although extracts from BeWo cells also bound this fragment, they exhibited a different mobility (data not shown). Transfections in the Rcho-1 cell line also identified a sequence at −400 to −340 bp that increased expression. Gel shift analysis showed a factor present in Rcho-1 cells that bound this region and was specifically competed with excess unlabeled fragment (Fig. 6, *right*

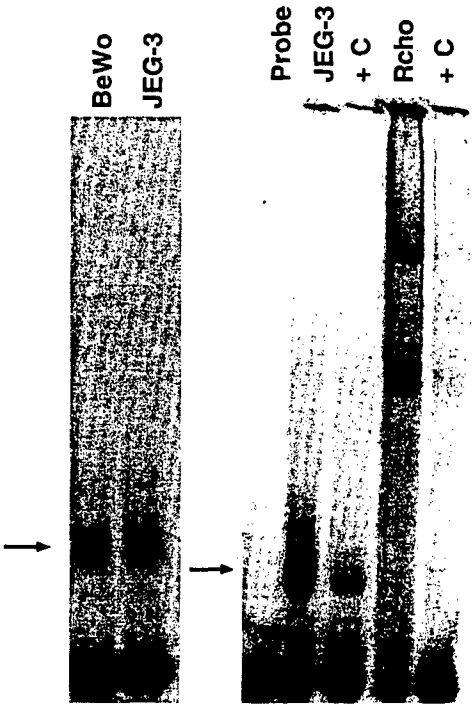


FIG. 5. Electromobility shift analyses of the hCRF promoter. The conditions for the assays are described in *Materials and Methods*. The *arrows* indicate the locations of the shifted bands of interest. The probe is from −150 to −68 bp of the hCRF gene. *Left panel*, Extracts from human choriocarcinoma cell lines, BeWo and JEG-3, display similar shifts. BeWo, Nuclear extract from BeWo cells; JEG-3, nuclear extract from JEG-3 cells. *Right panel*, Human and rodent choriocarcinoma cells display different shifts. Probe, Probe alone; JEG-3, extract from JEG-3 cells; +C, JEG-3 cell extract plus excess unlabeled competitor; Rcho, extract from Rcho-1 cells; +C, Rcho-1 cell extract plus excess unlabeled competitor.

*panel*). Whether this activator may participate in the regulation of other rodent-specific placental genes has yet to be determined.

## Discussion

CRF exhibits a complex pattern of expression and regulation. In the hypothalamus, CRF regulates the expression and release of ACTH from the anterior pituitary (1). CRF in the hypothalamus is expressed in a circadian pattern (35). It is subject to feedback inhibition at the level of transcription by glucocorticoids, the end product of the hypothalamic-pituitary-adrenal axis that CRF controls (36). CRF is also a key part of the stress response, with high levels of CRF stimulating increased levels of glucocorticoids, but in a manner that is not sensitive to feedback inhibition (1). CRF is expressed by T lymphocytes (3) and may itself act as a lymphokine, directly influencing the immune system independent of ACTH (10). Placental expression of CRF is primate specific (2), and CRF plays a role in timing the onset of human labor (5). Yet, the regulators of CRF expression in human trophoblasts and the mechanisms used by CRF to affect maternal-fetal physiology and parturition are not well understood. In placenta, expression of CRF increases throughout gestation (6) and is neither circadian nor feedback regulated by glucocorticoids (8). CRF is a single copy gene (37), and the

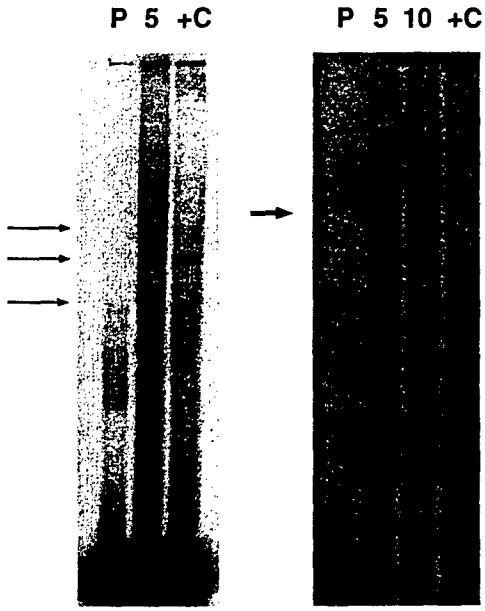


FIG. 6. Electromobility shift analyses of the hCRF promoter. The conditions for the assays are described in *Materials and Methods*. The *arrows* indicate the locations of the shifted bands of interest. *Left panel*, CRF repressor. The probe is from −532 to −400 bp of the hCRF gene. P, Probe alone; 5, 5 μg extract from Rcho-1 cells; +C, Rcho-1 cell extract plus excess unlabeled competitor. *Right panel*, Rodent CRF activator. The probe is from −400 to −340 bp of the hCRF gene. P, Probe alone; 5, 5 μg extract from Rcho-1 cells; 10, 10 μg extract from Rcho-1 cells; +C, 10 μg Rcho-1 cell extract plus excess unlabeled competitor.

peptide and proximal promoter sequences are highly conserved in the human, rat, and mouse genes (15, 38–40). Furthermore, CRF mRNA is identical in the human hypothalamus and placenta (41), suggesting that despite the differences in regulated expression, at least some promoter sequences may be used in both tissues.

There are currently no suitable cell culture systems available for detailed molecular studies of the regulation of CRF in hypothalamic neurons. However, there are several useful model cell lines for human placental trophoblasts. The JAR, JEG-3, and BeWo choriocarcinoma cell lines retain features of placental trophoblasts and have been successfully used for molecular studies of placental gene expression (19, 26–30). Recently, a corresponding rodent choriocarcinoma cell line, Rcho-1, has been established and used for expression studies (20). The combination of choriocarcinoma cell lines and CRF promoter fragments from both the human and mouse have provided the tools to directly compare the expressions of both genes in cells of human or rodent origin. Our experiments investigate the basis of species-specific expression of CRF by segregating the contributions of species-specific *trans*-acting factors from *cis*-acting sequences (Fig. 7). These results consistently demonstrate that *trans*-acting factors, differing between human and rodent choriocarcinoma cell lines, play a dominant role in dictating the species-specific expression pattern of CRF. Our results in these model systems reflect the results observed in placental trophoblasts. In human placenta, the hCRF gene is expressed, and in the human BeWo cell line model, hCRF reporter genes are expressed. In contrast, in rodent placenta, the CRF gene is not expressed, and in the Rcho-1 rodent cell line model, a mouse CRF reporter gene is not expressed. The experiments in which rodent and human reporter genes are expressed in cells derived from the heterologous species allow determination of the contributions of cellular *vs.* sequence differences. The human BeWo cells express a mouse CRF reporter gene, whereas rodent Rcho-1 cells do not express hCRF reporter genes. Therefore, *trans*-acting factors differing between human and rodent cells dominate in the observed species-specific CRF expression pattern in choriocarcinoma cells.

The results from these studies differ significantly from those reported for CG, which has served as a model for human-specific placental expression. The α-subunit of CG, like CRF, demonstrates a species-specific expression pattern. It is expressed in the placentas of humans, higher primates, and horses, whereas expression is restricted to the pituitary in other animal species (26). In humans and primates, tissue-specific expression of CGα results from a combination of *cis*-acting elements located in the 5′-proximal promoter region (26–29). In humans, two 18-bp direct repeats exist that contain a consensus cAMP response element (CRE), TGACGTCA (27, 28, 42, 43). The two repeats are absolutely necessary for cAMP responsiveness as well as tissue-specific expression (27, 28, 42, 43). Upon deleting these repeats, placenta-specific expression of a CAT reporter gene driven by the CGα promoter decreases to background levels (27). Upstream of the repeats, another *cis*-acting element exists that confers placenta-specific expression to a nonplacental heterologous promoter (27). It is known as the trophoblast specific element (TSE), and it binds a placenta-specific protein, TSEB (27, 29). If one deletes the CREs, the TSE loses its activity (27). Therefore, it appears that a protein-protein interaction occurs between a CRE-binding protein family member bound to the CREs and the TSEB, and the interaction is required for placenta-specific expression (28). In primates, placental expression of CGα depends on the presence of the TSE and either one or two copies of the 18-bp CRE (26). Thus, it appears that the species-specific expression of CGα in the placenta is very complex, relying on a combination of various *cis*-acting elements located upstream of the TATA box.

The species-specific expression of CRF, unlike that of CGα, cannot depend on the differences in CRE sequences, as functional CREs are conserved in human, mouse, and rat promoters (15, 30, 38, 44). Furthermore, our experiments comparing mouse and human CRF promoters in the choriocarcinoma cell lines confirm the dominant effect of species-specific *trans*-acting factors in determining the placental expression of CRF. Nonetheless, the CRE and the factors mediating this response appear to play a major role in CRF expression.

cAMP pathways play an important role in the biology of placental trophoblasts (34). In addition to the canonical sequences in CG and CRF, cAMP may play a critical role in the developmental pathway of trophoblasts, modulating both the morphological and biochemical changes that occur during the differentiation of cytotrophoblasts to syncytial trophoblasts (19). The induction of transcription factors by cAMP may play an important role in mediating its effects. Other endocrine genes including LHβ are regulated by cAMP, but seem to lack canonical CRE sequences (45). Our data demonstrate CRF induction via the CRE, but do not exclude the participation of additional sequences. We have

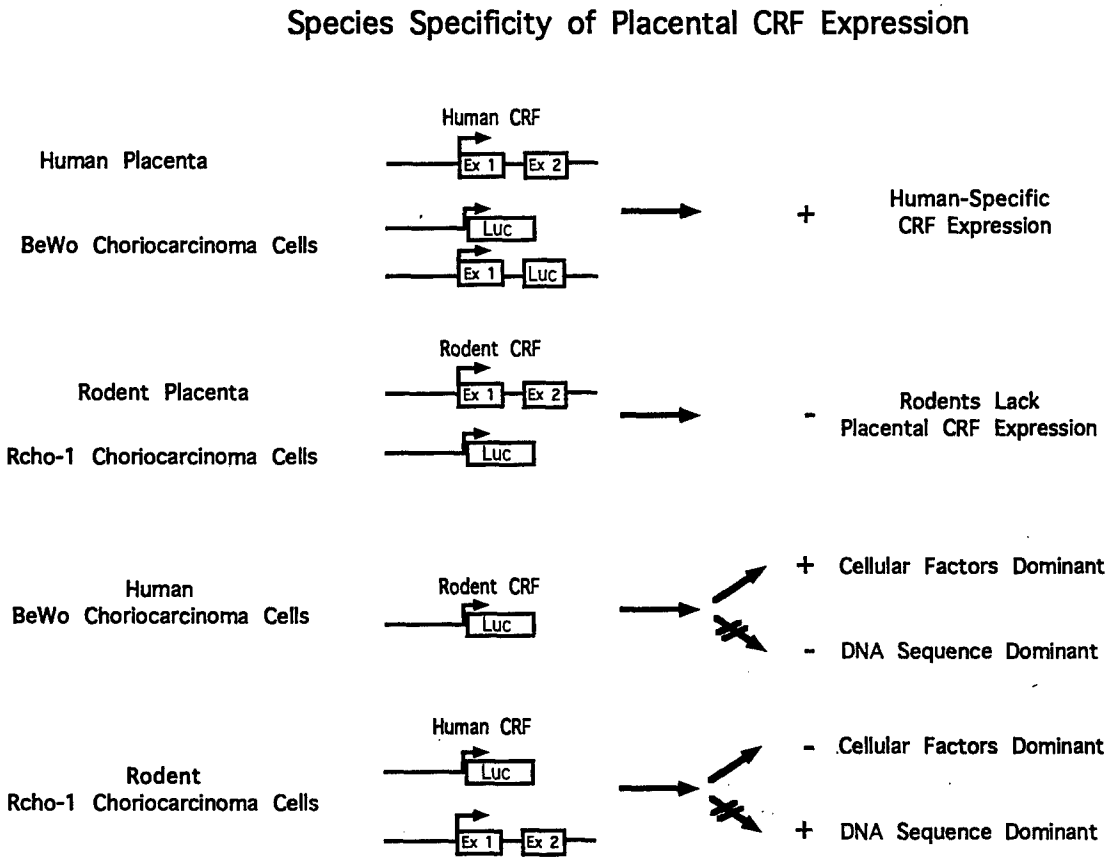


FIG. 7. Species specificity of placental CRF expression. The species specificity of CRF expression in placenta was modelled using human and rat choriocarcinoma cell lines and reporter genes derived from both human and mouse CRF genes. In human placenta and BeWo cells, hCRF genes are expressed (+). In rodent placenta and Rcho-1 cells, rodent CRF genes are not expressed (−). The basis of this species difference in CRF expression can be explained by either differences in cellular factors (Cellular Factors Dominant) or DNA sequence differences (DNA Sequence Dominant). Introduction of human and rodent CRF reporter genes into cells from the other species distinguishes these two possibilities. The results presented above are consistent with the former alternative in determining species-specific CRF expression.

identified one such region proximal to −200 bp, and a corresponding candidate nuclear binding factor in both BeWo and JEG-3 cells. We have no information yet whether this non-CRE-mediated activation may reflect synergy, modification, or activation of preexisting factors, or whether cAMP initiates a transcription cascade ultimately leading to the production of new transcriptionally active protein factors.

Two additional sites were located in the CRF 5′-flanking promoter that are involved in regulating CRF expression. A repressive sequence from −532 to −400 bp prevents the expression of CRF in Rcho-1 cells. The effect of this sequence is on basal expression. This indicates that the repression of CRF in rodent trophoblasts cannot be solely due to differences in the ability of Rcho-1 cells to respond to forskolin and 8-Br-cAMP, but also involves distinct *trans*-acting factors separate from these responses. We have identified a candidate nuclear factor that binds to this region that is present in Rcho-1 cells. In addition to the repressive element, a positive *cis*-acting element was identified within the region from −400 to −340 bp. The ability of this element to increase the basal expression of CRF in the rodent trophoblast is unmasked by removal of the upstream repressor sequences. Although the factor interacting with this sequence does not mediate expression of the full CRF promoter, a role for this factor in the regulation of other rodent trophoblast genes has not yet been investigated.

The participation of all of these CRF sequences and factors in central nervous system expression in paraventricular hypothalamic parvocellular neurons or the activity of potential repressors in non-CRF-expressing neurons remains to be determined. Also, the identities of the trophoblast factors that bind to these sequences and their potential roles in the regulation of other placental genes and in human development and parturition remain to be elucidated by future studies.

## Acknowledgments

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# Characterization of a Human-Specific Regulator of Placental Corticotropin-Releasing Hormone

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**The hypothalamic hormone CRH is also expressed in the placentas of humans and higher primates and may play an important role in the regulation of labor. In choriocarcinoma cell lines, activation of cAMP-dependent pathways increases human (h)CRH reporter gene expression. A cAMP-responsive region distinct from the cAMP response element at −220 bp, has been identified between −200 and −99 bp, and a candidate transcription factor was identified in nuclear extracts of human, but not rodent, choriocarcinoma cell lines.**

**This region, which does not contain a canonical cAMP response element (CRE), transfers protein kinase A responsiveness to a heterologous promoter. Electromobility shift assays and methylation and uracil interference studies localized factor binding to a 20-bp region from −128 to −109 bp of the hCRH promoter. This 20-bp fragment exhibited a similar shift in nuclear extracts from both human term placenta and from human JEG-3 cells. Base contacts, identified in interference studies, were confirmed as critical for binding, as a mutation of these bases abolished factor binding. Furthermore, a CRH promoter containing this mutation exhibited a diminished response to forskolin. UV cross-linking demonstrated the protein in nuclear extracts from human, but not rodent, choriocarcinoma cell lines and estimated its size as 58 kDa. Although this factor participates in cAMP-regulated gene expression, competition electrophoretic mobility assays demonstrated that the factor does not bind to a CRE. Furthermore, neither anti-CREB nor anti-ATF2 antibodies alter factor binding. These data identify this 58-kDa protein as the human-specific CRH activator previously identified as a candidate factor contributing to the species-specific expression of CRH in human placenta. (Molecular Endocrinology 12: 1228–1240, 1998)**

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## INTRODUCTION

CRH is a 41-amino acid neuropeptide hormone that regulates the hypothalamic-pituitary-adrenal (HPA) axis. It is highly conserved and is primarily expressed in the parvocellular neurons of the paraventricular nucleus of the hypothalamus. Secretion of CRH into the hypophyseal portal blood system activates the synthesis and secretion of ACTH from the anterior pituitary corticotrophs. ACTH, in turn, activates the synthesis and secretion of cortisol from the adrenal glands. Cortisol modulates this pathway via an inhibitory feedback loop, affecting both CRH and ACTH. Normally, CRH is expressed in a circadian pattern; however, during periods of stress, CRH continues to be expressed, in spite of the high levels of circulating glucocorticoids (1). The regulation of this HPA pathway is primarily transcriptional. Some neuronal transcription factors, including the CREB/ATF and POU-homeo transcription factor families, have been identified as participating in the regulation of this complex neuroendocrine system (2–6). Yet, we still lack a complete understanding of the molecular mechanisms responsible for circadian expression, the stress response, and glucocorticoid feedback.

CRH is expressed at other sites in the central nervous system and in peripheral organs. In primates, the placenta produces the highest concentration of CRH outside of the hypothalamus (1), while other animal species, including rats, mice, and guinea pigs fail to express CRH in their placenta (7, 8). Recent studies indicate that placental CRH may serve as a key component in timing the onset of human labor (9). Placental CRH is identical to the peptide synthesized and secreted in the nervous system, and CRH is a single copy gene (1). Thus, the expression of placental CRH in humans and high primates, and not in other species, indicates that unique mechanisms, distinct from those controlling hypothalamic expression, must control expression in placenta.

Our previous studies have investigated the molecular mechanisms controlling this species-specific placental expression of CRH. By comparing the activity of mouse and human CRH promoters in human and rodent trophoblast cell lines, we established that cellular differences, rather than DNA sequence differences, play the dominant role in establishing the species-specific expression pattern (10). Using nuclear extracts from the rodent and human cell lines, we identified three species-specific candidate *trans*-acting factors (10).

In our earlier studies, the ability to express CRH with both tissue and species specificity appeared to be linked to cellular cAMP responsiveness. In addition to the highly conserved canonical cAMP response element (CRE) located at −220 bp (2, 11), we identified an additional cAMP-responsive region in the human CRH promoter from −200 to −99 bp, which does not contain a canonical cAMP-regulatory site. One of the candidate *trans*-acting factors binds to this region, and it is present in nuclear extracts from the human, but not rodent, choriocarcinoma cell lines (10). The aim of the current study was to further characterize this protein factor and its DNA binding site and to confirm its participation in placental expression of CRH.

## RESULTS

### Fine Mapping of the Regulatory Region

Our previous studies in cell culture systems indicated that the expression of CRH in placenta is controlled by species-specific *trans*-acting factors (10). Transient transfections using deletions of the hCRH promoter identified a cAMP-responsive regulatory region between −200 and −99 bp, distinct from the canonical CRE at −220 bp. Examination of this region revealed that it does not contain a classic CRE. A human-specific factor that bound to this region was present in nuclear extracts from human choriocarcinoma cell lines (10).

To more precisely define the binding site for this human-specific factor, nuclear extracts from JEG-3 cells were used in a series of electrophoretic mobility assays (EMSAs), summarized in Fig. 1. The DNA fragments used in these analyses included portions of the hCRH proximal promoter spanning the region from −196 to −73 bp. Initially, two labeled fragments, −196 to −136 bp and −146 to −73 bp, had been used to divide this region approximately in half. The candidate nuclear factor bound only to the fragment from −146 to −73 bp (10). Next, to further define the location of

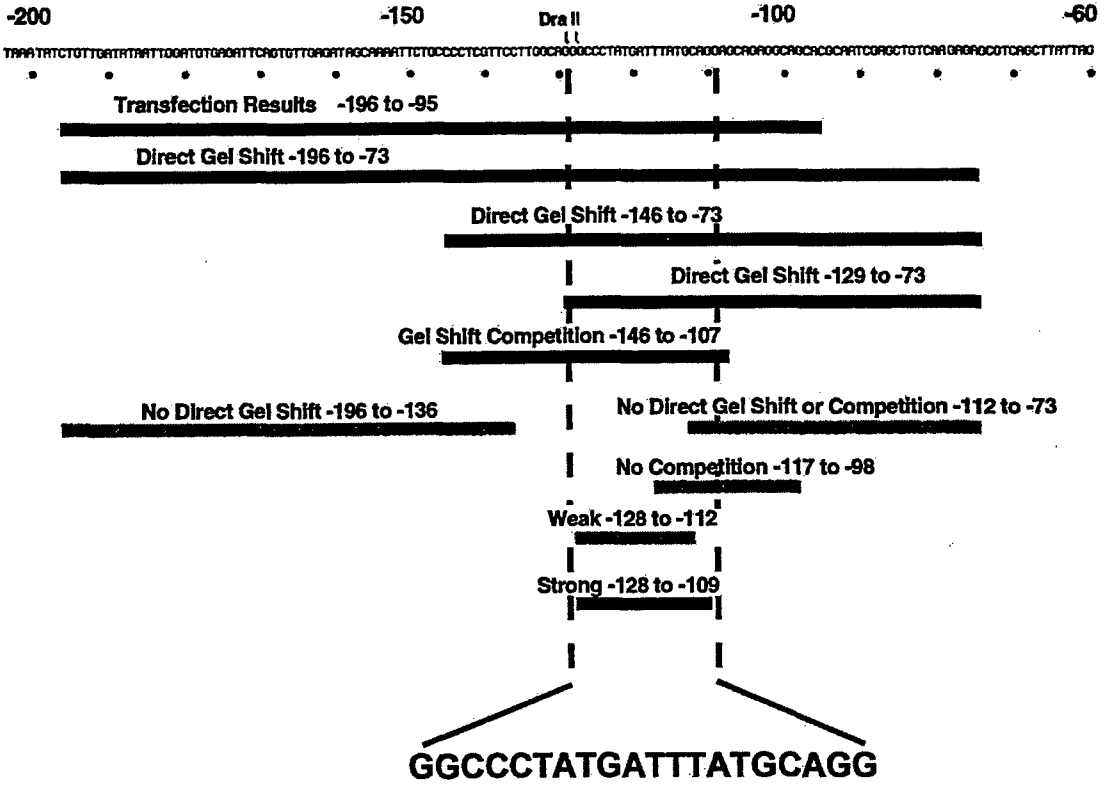


**Fig. 1.** Location of the Human-Specific Nuclear Factor DNA Binding Site

The DNA sequence for the human CRH promoter from −200 to −60 bp is shown for reference. Results obtained from transfection and EMSAs are indicated for each fragment. The 20-bp binding site identified by these analyses is from −128 to −109 bp in the hCRH promoter and is dGGCCCTATGATTTATGCAGG.

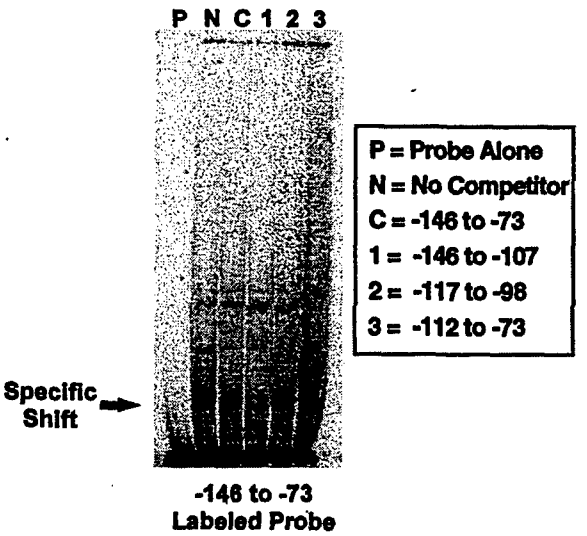


**Fig. 2.** Competition Studies Map the Location of the Human-Specific Nuclear Factor DNA Binding Site

JEG-3 nuclear extract (23 μg) was incubated with the labeled probe, −146 to −73 bp hCRH, in the absence or presence of excess (1 pmol) unlabeled fragments as indicated. The *arrow* indicates the location of the complex.

the binding site, unlabeled oligonucleotide pairs were used as competitors in EMSA with the −146 to −73 labeled hCRH fragment. A 40-bp oligonucleotide duplex from −146 to −107 bp of the hCRH promoter specifically competed the shifted band created by the candidate nuclear factor (Fig. 2). Two other oligonucleotides, −117 to −98 bp and −112 to −73 bp, did not compete with the probe for the binding of the candidate nuclear factor (Fig. 2).

One of the characteristics of an independent regulatory element is the ability to transfer regulation to a heterologous promoter. The region from −146 to −107 bp was further analyzed to determine whether this 40- bp sequence and the corresponding DNA binding factor identified *in vitro* retained the cAMP responsiveness originally associated with the larger region of the promoter. The 40-bp oligo duplex from −146 to −107 bp of hCRH was multimerized as three and six copies and inserted in front of a minimal 36-bp promoter, p36 (12). These reporter genes were cotransfected into the JEG-3 cells along with either the control plasmid, Rous sarcoma virus (RSV)-Neo, or with the protein kinase A (PKA) catalytic subunit β, RSV-PKA. Responsiveness to PKA pathways was determined as the fold increase in activity by comparing the expression with RSV-PKA to that with the RSV-Neo control. As shown in Fig. 3, addition of three or six copies of the 40-mer region progressively increased the PKA responsiveness of the p36 minimal promoter. The results from these experiments indicated that the region from −146 to −107 bp of the hCRH promoter was sufficient for cAMP responsiveness and transferred this responsiveness to a heterologous promoter.

Having confirmed that this smaller region of the promoter still retained regulatory activity, additional EMSAs were performed to determine a minimal binding site. The −146 to −73-bp fragment of the hCRH promoter contains *Dra*II sites centered at −130 and −129 bp (Fig. 1). Digestion of this region with *Dra*II generated two fragments for use in EMSA, but only the proximal fragment from −129 to −73 bp was bound by nuclear extracts (Fig. 1). These results narrowed the potential nuclear factor binding site to a 17-bp region from −128 to −112 within the hCRH promoter. A 17-bp oligonucleotide duplex was created, which corresponds to this potential site. It was used in EMSA as a labeled probe (Fig. 4). The candidate nuclear factor bound this fragment, but only weakly. Also, this sequence was a weak and inconsistent competitor for factor binding to the −146 to −73-bp probe (data not shown).

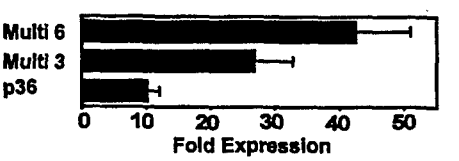


**Fig. 3.** Transfer of cAMP Responsiveness to a Heterologous Promoter

Human JEG-3 cells were cotransfected with the indicated reporters and either RSV-Neo or RSV-PKA. Fold expression is the relative increase in luciferase activity due to the expression and activity of the PKA catalytic subunit β for each promoter construct, compared with the RSV-Neo control. p36 is a minimal promoter. Multi 3 and Multi 6 are p36 minimal promoters with three and six copies of the −146 to −107 hCRH oligonucleotide, respectively. Data shown are means ± SEM from five experiments.

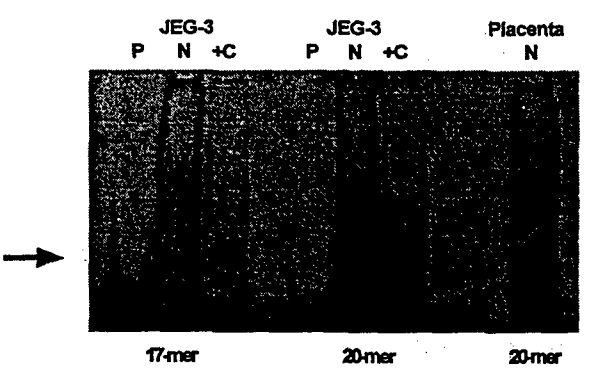


**Fig. 4.** Purified JEG-3 Nuclear Extract and Crude Placental Nuclear Extract Bind to the 20-mer Sequence

EMSAs were performed using either the 17-mer sequence (−128 to −112 hCRH) or the 20-mer sequence (−128 to −109). Reactions contained partially purified JEG-3 nuclear extract (2.6 μg) or crude human term placental nuclear extract (1 μg). When indicated, 1 pmol excess unlabeled 20-mer competitor DNA was present. P, Probe alone; N, nuclear extract; +C, nuclear extract plus competitor. *Arrow* indicates specific complex.

### Methylation and Uracil Interference Analysis

Because some of the fragments used in these studies may have split the binding site, the precise boundaries of the binding site were not certain. To more clearly identify the residues involved in binding, interference assays were performed. These assays identify DNA bases that, when modified, interfere with the binding of the nuclear factor to the hCRH promoter fragment (13). The labeled fragment used in these assays was the hCRH proximal promoter fragment from −146 to −73 bp.

For the uracil interference analysis, end-labeled hCRH fragments were generated to contain partial substitutions of deoxyuracil for thymine residues. Because crude nuclear preparations contained nuclease activity that degraded deoxyuracil-substituted DNA (S. Adler, unpublished results), binding reactions were performed using partially purified JEG-3 nuclear extracts. The results from these assays revealed that replacing the thymine with deoxyuracil at positions −121, −118, −116, and −114 on the sense strand, and at −111 on the antisense strand, interfered with the ability of the candidate nuclear factor to bind to the fragment (Fig. 5).

For the methylation interference analysis, end-labeled hCRH fragments were partially methylated using dimethyl sulfate. These experiments identified the guanines at positions −120 and −113 on the sense strand and at −112 on the antisense strand as residues critical for binding (Fig. 5).

These results from the interference assays clarified our initial mapping by EMSA. Interference assays identified the dA/T base pair at −111 of the hCRH proximal promoter as being important for binding (Fig. 5). Deletion analysis did not have the resolution to clearly identify this base pair, and it is not included in the weakly active 17-mer fragment, −128 to −112 bp. Therefore, a 20-mer from −128 to −109 bp of hCRH was synthesized. The nuclear factor bound this oligonucleotide (Fig. 4). The 20-mer also effectively competed the bound factor from the −146 to −73 labeled hCRH fragment (Fig. 6B). Therefore, the DNA-binding site for the human-specific nuclear factor was defined as −128 to −109 bp within the hCRH proximal promoter.

### Mutation of the DNA-Binding Site

Our identification of a minimal binding site for the human nuclear factor relied on *in vitro* binding studies. Before more functional studies, we performed site-directed mutagenesis to alter specific critical residues in the binding site. Two regions identified by interfer-

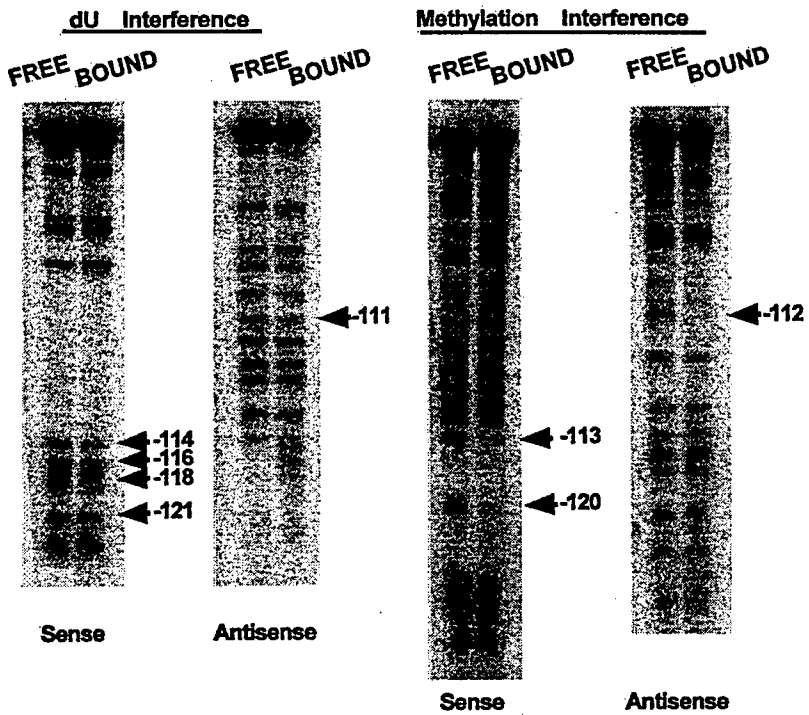


**Fig. 5.** Uracil Interference and Methylation Interference

Partially purified JEG-3 nuclear extract was incubated with the hCRH fragment from −146 to −73 bp, which had been end labeled on either the sense or antisense strand. For deoxyuracil interference, the fragments were generated to contain partial substitution of deoxyuracil for thymidine residues. For methylation interference, the fragments were partially methylated using dimethyl sulfate. The DNA bases that when modified affected the binding of the protein are noted with *arrows*, and their location in the hCRH promoter is given. Free, Probe not bound by extract; bound, probe bound by extract in EMSA. The end-labeled strands are either sense or antisense, as indicated.

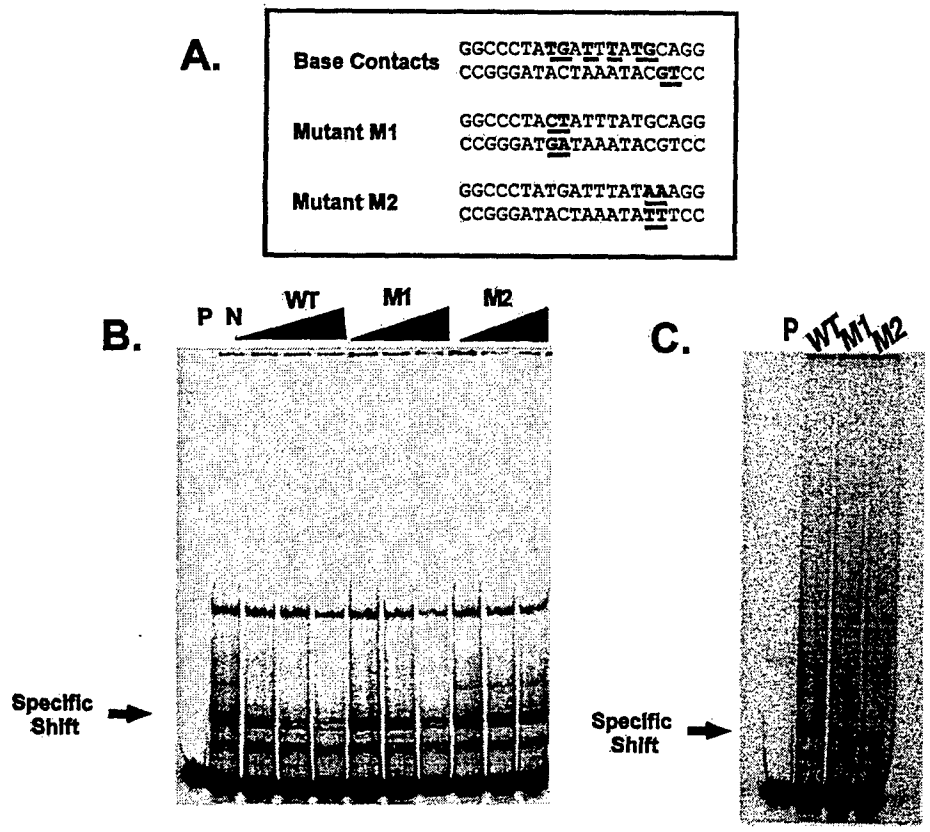


**Fig. 6.** Mutation of the Human-Specific Factor DNA Binding Site Alters Binding of the Protein in EMSAs

A, The 20-bp binding site sequence is shown with base contacts indicated in *bold and underlined*. The M1 mutation contains a change in the hCRH nucleotides at positions −121 and −120 from dTG to dCT, shown in *bold and underlined*. The M2 mutation contains a change in the hCRH nucleotides at positions −113 and −112 from dGC to dAA, shown in *bold and underlined*. B, Competition with native and mutant 20-mer binding sites from −128 to −109 bp in the hCRH promoter. JEG-3 nuclear extract (20 μg) was incubated with labeled probe (−146 to −73 hCRH) in the absence or presence of excess unlabeled 20-mer fragments. P, Probe only; N, no competitor; Crescendo, increasing quantities (1 pmol, 3 pmol, 6 pmol) of unlabeled wild type (WT) 20-mer fragment, M1 mutant fragment, or M2 mutant fragment. C, Direct binding EMSA of mutant 20-mer sequences. The labeled fragments used in these EMSAs were −146 to −73 of the hCRH promoter (wild-type, M1, or M2) and were incubated with JEG-3 nuclear extract (20 μg), as indicated. The *arrow* indicates the location of the complex. P, Wild type probe alone; WT, wild-type probe with nuclear extract; M1, probe with M1 mutation with nuclear extract; M2, probe with M2 mutation with nuclear extract.

ence studies were selected. The dT at −121 and the dG at −120 on the sense strand of hCRH were changed to dC at −121 and to dT at −120 (mutation M1, Fig. 6). The dG at −113 and the dC at −112 on the sense strand of hCRH were both changed to dA (mutation M2, Fig. 6). These mutant hCRH fragments were used to generate labeled DNA fragments from −146 to −73 of the hCRH proximal promoter. The mutant probes were compared with the wild-type −146 to −73 hCRH fragment in an EMSA (Fig. 6C). The human-specific nuclear factor bound to the wild-type fragment and to the fragment with the M1 mutation, although binding was weak when compared with the wild-type fragment. Introducing the M2 mutation into the labeled fragment completely eliminated the ability of the nuclear factor to bind to its site. These results were confirmed by using an oligonucleotide duplex from −128 to −109 of the hCRH promoter that contains the above mutations in EMSA as unlabeled cold competitors (Fig. 6B). These analyses confirmed that the M1 mutation was a weak competitor, while the M2 mutation was unable to compete for binding of the nuclear factor. These mutations thus confirm the identification of the binding site and the DNA base contacts critical for nuclear factor binding.

### Transfection Studies

The −532 CRH promoter was examined for sequences similar to the 20-mer binding site, and no other sites were found (data not shown). However, a partial homology was found in the luciferase coding region, and the corresponding 20-mer oligonucleotide exhibited weak binding on EMSA (data not shown). This site was mutated to a sequence similar to the M2 mutation, a change of one amino acid, Ser 399 to Lys. This mutation did not affect the activity of the *in vitro* translated luciferase enzyme (data not shown). To eliminate the possible contributions of the homology in

the luciferase gene, this mutated luciferase gene was used as the reporter in the subsequent experiments.

The M2 mutation in the binding site of the nuclear factor provided a way to further characterize the role of the placental nuclear factor by analyzing the functional consequences of factor binding on the placental expression of CRH. Transfections in Bewo cells were performed to compare the activity of the −532 CRH promoter to the activity of a promoter containing the M2 mutation, a promoter with the canonical CRE mutated (XCRE) (10), or a promoter containing both XCRE and M2 mutations (Fig. 7). The M2 mutation alone gave little reproducible change in forskolin responsiveness of the promoter in the presence of the intact CRE. However, when the CRE was mutated, the M2 mutation further decreased forskolin responsiveness of the CRH promoter.

### Identification of the Nuclear Factor as a 58-kDa Protein

Our previous experiments identified the DNA-binding site of the nuclear factor within the CRH promoter. In addition, experiments also linked factor binding to PKA-regulated induction of CRH expression. More information about the protein was determined by using UV-cross-linking.

The molecular mass of a DNA-binding protein can be estimated by using UV light to cross-link the protein to a labeled DNA fragment that contains the protein's DNA-binding site. The cross-linking reaction is facilitated by incorporating bromodeoxyuridine (BrdU) into a labeled DNA fragment containing the binding site. After UV irradiation and nuclease digestion, the protein can be resolved on SDS/PAGE and identified by autoradiography. The migration of the protein relative to the migration of known mol wt standards allows an approximate molecular mass of DNA binding subunits to be determined (13). Using this method, nuclear extracts from the human choriocarcinoma cell lines, JEG-3 and BeWo, and from the rodent choriocarcinoma cell line Rcho-1, were incubated with a labeled, BrdU-containing fragment from −146 to −73 of the hCRH promoter. Binding reactions were exposed to UV, and bound proteins were resolved on SDS/PAGE (Fig. 8). A single band was present with the JEG-3 and BeWo extracts. Furthermore, this band was specifically competed by excess cold competitor when included in the binding reactions. In addition, the band was not present with the Rcho-1 nuclear extract. By comparing the migration of the identified labeled band to the migration of the known molecular mass standards, we estimated the molecular mass of the human-specific nuclear factor as 58 kDa.

### The Nuclear Factor Does Not Bind to a CRE and Does Not Contain CREB or ATF-2

The data presented above indicate that the factor we identified binds to a sequence unrelated to the canonical CRE and that its estimated subunit molecular mass is distinct from that of CREB, the 43-kDa transcription factor most widely associated with cAMP transcriptional regulation. To further distinguish the identity of the placental factor from CREB, we evaluated the ability of the canonical CRE sequence to compete for binding in EMSA. Figure 9 shows that while the 20-mer binding site oligonucleotide competes for binding with the −146 to −73 probe, neither the canonical CRE nor a canonical nuclear factor (NF)-κB binding site competes in the gel shift assay.

To further exclude the possibility that the placental factor was identical to CREB, or contained a member of the CREB family in a complex, we performed supershift EMSA with antibody preparations either directed against CREB or the related factor, ATF-2. ATF-2 is a transcription factor related to CREB, which at 56 kDa, is similar in size to the placental factor. Figure 10A shows that appropriate commercial antibodies produce a distinctive supershift in assays using preparations of ATF-2 and CREB, and a labeled DNA duplex probe containing the canonical CRE. Reticulocyte lysates appropriately programmed to translate ATF-2, but not a mock-translated preparation, produced a robust gel shift that was specific for binding to the CRE. The presence of specific anti-ATF-2 antibody

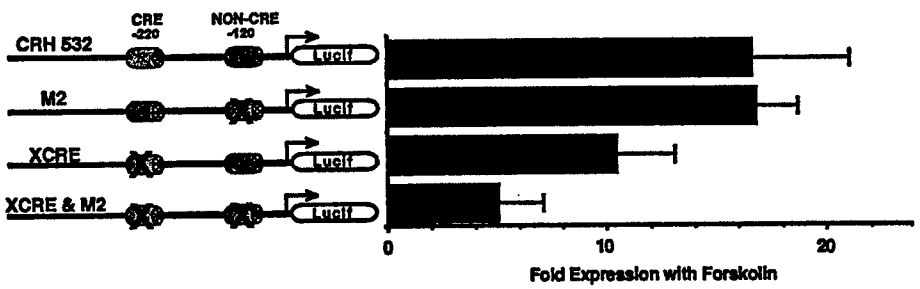


**Fig. 7.** Mutation of the Binding Site Reduces Forskolin Responsiveness of the hCRH Promoter

Human BeWo cells were transfected with the CRH −532 promoters, wild type or mutated as indicated, and responsiveness to forskolin was determined. Results are shown as fold expression and are the means ± SEM from three experiments. CRH 532, wild-type promoter; M2, CRH −532 promoter containing the M2 mutation; XCRE, CRH −532 XCRE promoter with mutation of the canonical CRE at −220 bp; XCRE & M2, CRH −532 double mutant.

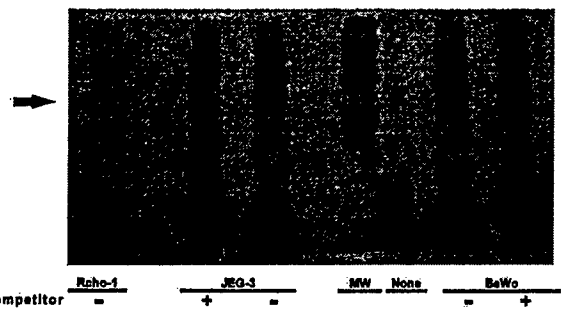


**Fig. 8.** UV Cross-Linking Studies Reveal the Human-Specific Factor to be a 58-kDa DNA Binding Protein

The labeled bromodeoxyuridine (BrdU) fragment used in this assay was from −146 to −73 bp in the hCRH promoter and was incubated with nuclear extracts from Rcho-1, JEG-3, or BeWo cells, with or without 10 pmol unlabeled −146 to −73 bp hCRH competitor, as indicated. The *arrow* indicates the location of the 58-kDa protein. MW, $^{14}$C-labeled protein mol wt standards; None, labeled BrdU fragment without nuclear extract.

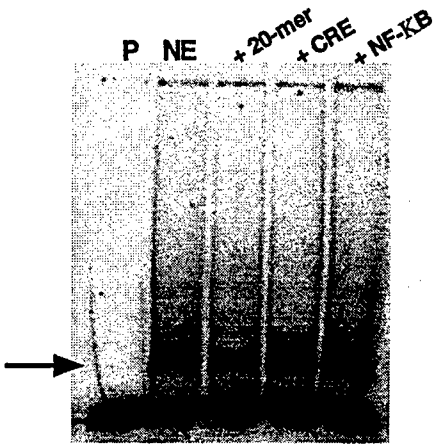


**Fig. 9.** The CRE Does Not Compete with Factor Binding

EMSA binding reactions contained JEG-3 nuclear extract (12.6 μg) and labeled −146 to −73 hCRH probe. Excess competitor (3 pmol) was added as indicated. P, Probe alone; NE, JEG-3 nuclear extract without competitor; + 20-mer, excess unlabeled 20-mer fragment; +CRE, excess unlabeled canonical CRE fragment; +NF-KB, excess unlabeled NF-KB binding site.

produced a diagnostic supershift. For similar experiments with CREB-specific antibody, nuclear extracts from a neuroblastoma cell line, BE(2)-C, and from Hela cells, were used. Specific binding to the CRE probe was dependent on the presence of nuclear extract and was competed by unlabeled CRE competitior. A diagnostic supershift was obtained upon addition of the anti-CREB antibody for BE(2)-C cell extracts, and for Hela cell extracts. In contrast to the supershift observed with preparations containing ATF-2 and CREB, Fig. 10C shows neither supershift nor diminished intensity of placental factor binding to the −146 to −73 DNA probe in the presence of anti-CREB or anti-ATF-2 antibodies.

### The Factor Is Present in Nuclear Extracts from Human Term Placenta but Not Hela Cells

Our previous studies (10) and the data presented above have demonstrated that the factor binding to the hCRH 20-mer sequence within the −146 to −73 sequence is present in human choriocarcinoma cell lines, but absent from the corresponding rodent choriocarcinoma cell line. To determine whether the factor might be present in another human cell line, EMSA were performed using nuclear extracts prepared from Hela cells, a human cell line that is not derived from placental choriocarcinoma cells. EMSA failed to demonstrate the presence of the distinctive shift seen with similar extracts from JEG-3 cells (Fig. 10B). The lack of EMSA activity in this Hela extract is specific for the placental factor, since this same Hela extract demonstrates activity in supershift EMSA for CREB activity (Fig. 10A).

In addition, we examined nuclear extracts from human term placentas for the presence of the factor using EMSA and the specific 20-mer DNA duplex as probe. As seen in Fig. 4, crude nuclear extracts from human placenta exhibit the characteristic shift seen with the partially purified extracts prepared from JEG-3 cells. These data provide further evidence for the specific expression of this factor in human placenta.

## DISCUSSION

CRH is a single-copy gene that is highly conserved and that displays a unique pattern of expression. Its primary site of expression is the hypothalamus, where it displays a circadian expression pattern, but one that is modulated by stress and by feedback regulation. It is also expressed in several peripheral tissues including placenta (1). The expression in placenta is uniquely species-specific. Only humans and high primates express the gene in this organ (7). This placental expression may reflect the unique changes in gestation and parturition that have occurred in human evolution. Furthermore, the mechanisms controlling the expression of CRH in the placenta must be distinct from those controlling the expression of CRH in the HPA axis, which are highly conserved across many species (1).

One other placental gene that has been extensively studied is the α- subunit of the glycoprotein CG. It has a central neuroendocrine role as an anterior pituitary peptide. It also has a species-specific expression pattern in placenta, with expression in primates and horses. For α-CG, evolutionary changes in *cis*-acting sequences within the promoter of the gene dictate the species-specific expression in placenta. The presence of one or two copies of a CRE is essential for α-CG expression, and minor changes in this *cis*-acting element result in the loss of expression in placenta (14–

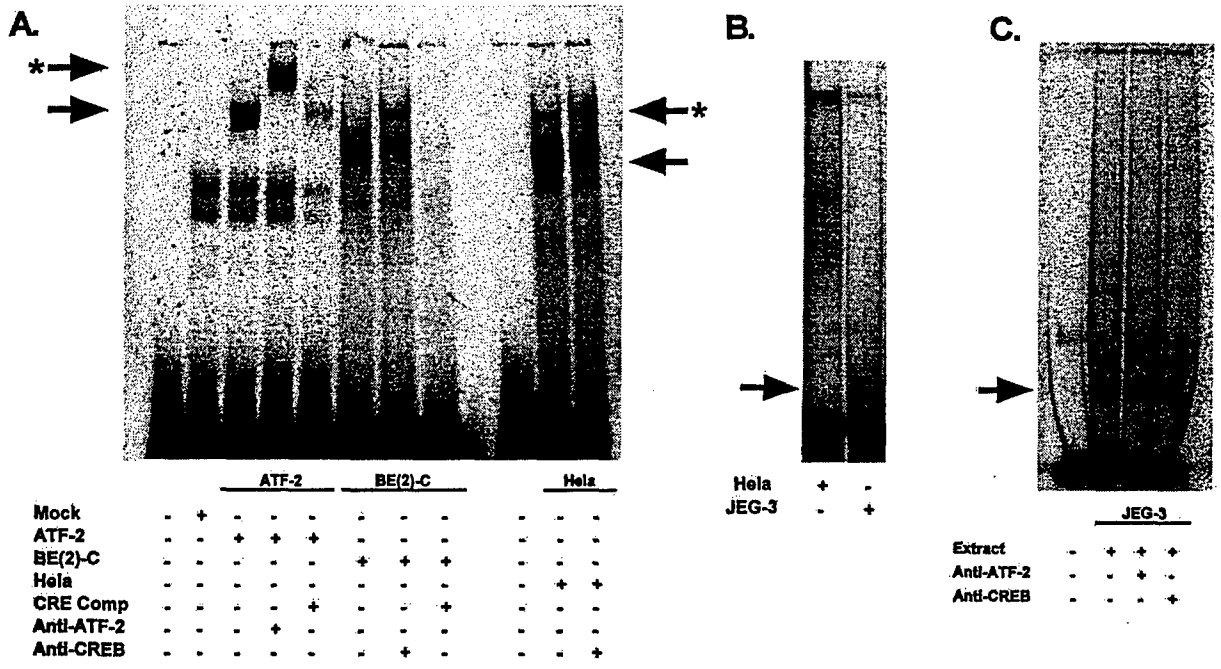


**Fig. 10.** The Placental Factor Is Not CREB or ATF-2 and Is Absent From Hela Cell Extracts

A, Specific antibodies produce distinct supershifts with ATF-2 and CREB. Supershift EMSAs were performed using a labeled CRE oligonucleotide as probe and antibodies against ATF-2 and CREB. Shift and supershift (*) are indicated by *arrows* for ATF-2 (*left*) and CREB (*right*). The addition of mock-translated lysate, translated ATF-2, BE(2)-C nuclear extract (15 μg), or Hela nuclear extract (5.3 μg) are shown. Anti-ATF-2 antibody, anti-CREB antibody, or 1 pmol unlabeled CRE oligonucleotide additions are as indicated. B, Hela nuclear extract lacks the placental shift. EMSA was performed with either Hela nuclear extract (12.6 μg) or JEG-3 nuclear extract (12.6 μg) using labeled probe (−146 to −73 hCRH). The *arrow* indicates the position of the placental-specific shift. C, Antibodies to ATF-2 and CREB do not affect placental factor binding. EMSA was performed with JEG-3 nuclear extract (12.6 μg) using labeled probe (−146 to −73 hCRH). Addition of anti-ATF-2 or anti-CREB antibody is as indicated. The *arrow* indicates the position of the placental-specific shift.

16). In addition, the *cis*-acting sequence, TSE (tissue specific element), contributes to the tissue-specific expression of α-CG (16–18). Experiments in transgenic mice have shown that the bovine α-CG promoter is expressed only in pituitary, while a transgene derived from the human promoter is expressed both in pituitary and placenta (15). It is therefore a combination and alteration of these *cis*-acting elements, and not differences in *trans*-acting factors, that play the dominant role in species-specific expression of α-CG in placenta.

Our studies present a different paradigm to explain the species-specific expression of CRH in placenta. Transfection studies identified three regions of the human CRH promoter that contributed to the expression pattern of CRH in placental trophoblasts. *In vitro* studies identified candidate nuclear factors binding to the regions targeted by our transfection analyses. Interestingly, these nuclear factors are species-specific. A candidate rodent repressor and activator were identified in rodent trophoblasts, and a candidate human-specific factor was identified in human cells (10). These results demonstrate that differences in *trans*-acting factors, not *cis*-acting sequences, are dominant in determining the expression of CRH in placental trophoblasts.

In human trophoblast cells, cAMP plays a critical role in both cell differentiation and gene expression (19, 20). Many genes in these cells, including both α-CG and CRH, are regulated by cAMP (2, 3, 21, 22). The results from our earlier studies implied that it was the ability of a cell to respond to cAMP that determines both the tissue and species-specific expression of CRH (10). CRH has within its proximal promoter a highly conserved classic CRE where members of the CREB/ATF family of transcription factors can bind and activate CRH gene expression (2, 11, 23). Our studies indicated that after mutational inactivation of the canonical CRE, cAMP responsiveness was retained. Deletional mapping identified a proximal cAMP-responsive region within the human CRH promoter from −200 to −99 bp, which does not contain any characterized classic cAMP-response elements (10). The human-specific factor binds to hCRH within this region, and fine mapping studies defined its binding site to be from −128 to −109 bp within the hCRH promoter. In our current studies, transfection experiments using human choriocarcinoma cell lines confirmed the activity of this region and its relationship to cAMP-regulated pathways.

In transfection studies, the M2 mutation, in combination with the mutation of the CRE, still retained slight

activation by PKA pathways, even though the human-specific factor cannot bind to this mutated promoter *in vitro*. While part of this activity may be due to low levels of binding *in vivo*, other factors or binding sites might also be contributing to this regulation. Majzoub *et al.* (24) have also reported PKA responsiveness of the CRH promoter distinct from the canonical CRE. They identified a site between −112 and −98 bp in the hCRH promoter based on its similarity to a sequence found in the human enkephalin promoter (25).[1] The site is adjacent to the human-specific factor DNA-binding site, which we identified at −128 to −109 bp in the hCRH promoter, and the two sites overlap by 4 bp. We cannot rule out a combination of these two sites as contributing to the cAMP responsiveness of this region. In our footprinting analysis, we did not detect binding of a factor to the −117 to −103-bp promoter region; however, the extracts used in this analysis were partially purified and selected for enrichment of the human-specific factor binding at −128 to −109 bp. It is possible that using crude nuclear extracts, a footprint might also be detected in the −117 to −103-bp region. In addition, our earlier studies indicated the possibility that other distal PKA-regulated sites exist within the 5-kb human CRH promoter (10). The region from −200 to −99 bp contains the most proximal site we could detect by deletion analysis and may not be the best binding site for the factors mediating the PKA responsiveness of the hCRH promoter. The combination of several cAMP-regulated sites including the CRE, as well as the absence of transcriptional repressors, may ultimately contribute to the tissue and species-specific expression of CRH, as well as the regulated responses of CRH to environmental or developmental signals.

In these studies, we have identified a 58-kDa protein as the human-specific factor that binds to the hCRH cAMP-responsive site from −128 to −109 bp. The factor is specific to humans as we demonstrate its presence in the nuclear extracts of the human choriocarcinoma cell lines, but not in the rodent choriocarcinoma cell line. The lack of expression of the identified DNA binding subunit, along with the expression of a transcriptional repressor, is likely to contribute to the inability of the rodent trophoblast to express CRH. The identification of the 58-kDa protein in human trophoblasts by UV cross-linking does not exclude the possibility of other protein subunits that may be components of this transcription factor. Nor can we exclude a requirement for additional proteins that may be necessary to elicit a response to cAMP.

Additional EMSA and supershift experiments indicate that although the activity of this placental factor is linked to cAMP pathways, it is distinct from CREB and ATF-2. We have also extended our characterization of the specificity of expression of this factor to not only human choriocarcinoma cells, but also to human term placental tissue. Our data also now exclude expression not only from rodent choriocarcinoma cell lines, but also from the human nonplacental Hela cell line which we previously showed lacked cell type-specific expression of CRH reporter genes in transfection studies (10).

The unique placental expression of CRH in higher primates is consistent with a role for CRH in fetal gestation and labor, especially in humans (26). The expression of CRH in human placenta begins around the seventh week of gestation and increases throughout pregnancy. During the last 5 weeks of gestation, there is a significant increase in CRH expression within the placenta (27). Studies have correlated placental expression of CRH with the length of gestation. Elevations of CRH occur earlier in pregnancies complicated by preterm delivery, and the level of CRH is lower in pregnancies extending post term (9). Placental CRH may also cross into the fetal circulation and stimulate the fetal HPA axis, resulting in the increase in cortisol seen within the fetus during the last 5 weeks of gestation. The cortisol surge is necessary for the maturation of fetal organs, and thus may contribute to the fetal signal for initiating parturition (28).

In our model cell culture system, the activity of the 58-kDa protein, in both specific DNA binding in preparations of nuclear extracts and in mediation of a transcriptional response to cAMP, appears to vary with growth conditions and cell density (M. A. Mallon and C. D. Scatena, unpublished results). It is tempting to speculate that these changes in activity may parallel the changes occurring in the trophoblast at term that result in the increased expression of CRH in human placenta.

The changes that occur in human pregnancy at term may also have other implications in the interpretation of our data from cell lines. We have shown that the human choriocarcinoma cell lines express the CRH activator and exhibit appropriate tissue-specific expression of transfected CRH reporter genes. These data do not provide an explanation for the reported absent or inconsistent expression of the endogenous hCRH gene in choriocarcinoma cell lines. It is possible that in these cell lines the endogenous CRH gene is damaged, deleted, or has been rendered inaccessible for cellular transcription. It is also possible that the choriocarcinoma cells are more representative of a preterm trophoblast and that additional factors or activation signals present in placenta at term would result in even greater reporter gene expression or in expression of the endogenous hormone. Also, our preparations of nuclear extracts often display a doublet or split pattern on EMSA, although the presence and intensity of this pattern is variable among our preparations. Possible explanations for this pattern may include differences in protein modification, *e.g.* the degree of phosphorylation in response to activation of protein kinase A. Alternatively, rather than indicating the consequences of a regulatory event, these

[1] The sequence presented in the publication differs from the actual CRH sequence by a single nucleotide at −109 The change does not significantly alter the observed homology.

forms may be the result of proteolytic cleavage occurring during the preparation of our extracts. The demonstration of the characteristic shift of the CRH activator in a crude nuclear extract from human term placenta may provide both an abundant source of the activator protein and also allow extension of our molecular studies from cultured choriocarcinoma cells to authentic human trophoblasts that reflect the changes occurring at term. Further evaluation of these possibilities remain for future studies.

The role CRH plays in fetal gestation and parturition suggests a requirement for strict regulation of the CRH gene in placenta. Further characterization of the activator protein, including cloning of its gene and determination of the role cAMP plays in its expression and activity, could clarify the mechanisms of regulation and the role of CRH in the human placenta and in parturition.

## MATERIALS AND METHODS

### EMSA

The preparation of the JEG-3, BeWo, Hela, and Rcho-1 nuclear extracts and the binding and electrophoresis conditions for the human-specific factor were previously described (10, 29). Results were visualized using autoradiography or by storage screen analysis. Duplex DNA fragments were labeled using either direct incorporation of radioactive nucleotides during PCR, 3′-end labeling with Sequenase (USB Corp., Cleveland, OH) or Klenow fragment (Life Technologies, Gaithersburg, MD), or 5′-end labeling with T4 polynucleotide kinase. DNA fragments used included hCRH −196 to −73, hCRH −146 to −73, hCRH −129 to −73, hCRH −196 to −136, hCRH −112 to −73, hCRH −128 to −112, and hCRH −128 to −109. Unlabeled duplex DNA fragments for competition were prepared by annealing complementary pairs of synthetic oligonucleotides and, if necessary, end filling with either Klenow fragment or Sequenase and deoxynucleoside triphosphates. Unlabeled competitors were added at approximately 1 pmol, and included hCRH −146 to −107, hCRH −112 to −73, hCRH −117 to −98, hCRH −128 to −112, and hCRH −128 to −109, as well as duplex oligonucleotides containing canonical binding sites for CREB and NF-κB purchased from Promega (Madison, WI).

Mutated versions of the DNA fragment hCRH −146 to −73 were created by PCR, using plasmids containing the indicated mutation as template, and oligonucleotide primers hCRH −146 to −126 (forward) and hCRH −73 to −93 (reverse). To create the mutated DNA fragment −146 to −73 M1, the plasmid CRH −200 M1 pBKS II (−) was used as template. To create the mutated DNA fragment −146 to −73 M2, the plasmid CRH −200 M2 pBKS II (−) was used as template. Similar reactions were used to generated the wild-type probe hCRH −146 to −73. Mutant plasmids were created using site-directed mutagenesis as described below.

Discarded human placental tissue was obtained at deliveries from pregnant patients with normal uncomplicated term pregnancies and labor, in accordance with the requirements of the local Human Studies Committee. Human tissues were handled in compliance with the requirements of the local Biosafety Committee. Small portions of placental tissue were trimmed of connective tissue and quick-frozen in liquid nitrogen in the delivery suite. This frozen tissue was used to prepare nuclear extracts as described above for cultured cells.

### Cell Lines

BeWo, JEG-3, and Hela cell lines were obtained from American Type Culture Collection (Rockville, MD). The Rcho-1 cell line was the generous gift of M. Soares (30). The BE(2)-C cell line (31) was a generous gift from Dr. June Beidler (Memorial Sloan-Kettering Cancer Center, New York). The BE(2)-C cell line was grown at 37 C in a 5% $CO_2$ incubator and in MEM/F12 medium supplemented with 10% FBS (Intergen, Purchase, NY), 5% enriched calf serum (ECS, Gemini Bioproducts, Calabasas, CA), and 1× nonessential amino acids (Mediatech, Washington DC). BeWo cells were grown in 5% $CO_2$ in RPMI 1640 with 5% FBS and 5% ECS. JEG-3 cells were grown in 5% $CO_2$ in MEM with 5% FBS and 5% ECS. Rcho-1 cells were grown in 5% $CO_2$ in NCTC-135 with 5% FBS, 5% ECS, 0.4% glucose, 50 μM 2-mercaptoethanol, and 100 μM Na-pyruvate. Hela cells were grown in 10% $CO_2$ in DMEM with 5% FBS and 5% ECS. All the above growth media were supplemented with antibiotics. All cells are routinely surveyed for mycoplasma using a PCR method from Stratagene (La Jolla, CA).

### Site-Directed Mutagenesis

Oligonucleotide-directed mutagenesis was performed in phagemid vectors using minor modifications of the method of Kunkel (32). Deoxyuracil containing phagemid DNA was prepared from CRH −200 pBKSII(−), CRH −532 pBKSII(−), and CRH −532 XCRE pBKSII(−) (10). To create the M1 mutation, the oligonucleotide primer (antisense) used for the mutagenesis was dCCTGCATAAATAGTAGGGCC, which changes the hCRH nucleotides at positions −121 and −120 from dTG to dCT. To create the M2 mutation, the oligonucleotide primer (antisense) used for the mutagenesis was dCCTCTGCTCCTTTATAAATCATAGGG, which changes the hCRH nucleotides at positions −113 and −112 from dGC to dAA. In addition, the luciferase coding region from the LΔ2S reporter plasmid (10) was mutated to remove a partial homology to the CRH binding region. Deoxyuracil containing phagemid DNA was prepared from the corresponding luc XT pBKSII(−) plasmid and the oligonucleotide primer (antisense) used for mutagenesis was dGTTTACATAACCTTTCATAATCATAGG. This primer changes the nucleotide codon TCC (ser-399) to AAA (lys), a change in sequence equivalent to the change introduced by the M2 mutation. Luciferase activity of the mutant protein was confirmed by *in vitro* translation using the TNT reticulocyte lysate system (Promega, Madison, WI) and assaying the lysate for luciferase activity similar to our assays of cell extracts described below.

All mutations were confirmed by dideoxy DNA sequencing, and corresponding reporter plasmids were made by subcloning the mutated sequences using conventional techniques.

### Expression Vectors and Plasmids

The plasmid for expressing the catalytic subunit of protein kinase A, RSV-PKA, was obtained from Richard Maurer (33). The RSV-Neo plasmid expressing the neomycin phosphotransferase II gene is as previously described (34).

The construction of luciferase reporter genes using LΔ2S plasmids was previously described (10). To create reporters containing three and six copies of the oligonucleotide duplex from −146 to −107 bp of the hCRH proximal promoter, partially kinased duplexes were multimerized in three or six copies using DNA ligase. These multimerized fragments were subcloned in front of the minimal 36-bp promoter, p36 (12).

### Transfections

Transient transfections were performed using a calcium-phosphate method (35) in 6- or 12-well plates. The conditions

for the 6-well plates have been previously described (10). For a 12-well plate, minor changes to the protocol were made. Two days before transfection, 40,000 cells per well were seeded in 1 ml growth media. Transfections used a total 71 μl of the calcium phosphate solution containing 1.4 μg DNA prepared in the same proportions as previously detailed (10). Cells were harvested in 200 μl of a Triton lysis buffer, containing 50 mM Tris/2 (*N*-morpholino) ethane sulfonic acid (pH 7.8), 1 mM dithiothreitol (DTT), and 1% Triton X-100. The lysate was assayed for luciferase activity as previously described (36), using a Monolight 2010 luminometer (Analytical Luminescence Laboratories, San Diego, CA). β-Galactosidase assays were performed using chlorophenol red β-galactopyranoside (Boehringer Mannheim, Indianapolis, IN) as substrate (37) and an Anthos plate reader (Anthos Labtec Instruments, Salzburg, Austria) with Delta Soft II software (Bio Metallics, Princeton, NJ). Results (fold expression) are shown as means ± SEM. For comparisons one-way ANOVA and the unpaired two-tailed *t* test were applied using Primer of Biostatistics software (Windows v. 4.0, MacGraw Hill, St. Louis, MO). Significance was determined as $P < 0.05$.

### Methylation Interference and Uracil Interference

Nucleotides in the binding site for the human-specific activator were identified using minor modifications of standard protocols for methylation interference and uracil interference (13). Specific 5′-end labeled DNA fragments were prepared using PCR. Before PCR, either the forward or reverse oligonucleotide primer was end labeled with T4 polynucleotide kinase using $\gamma^{32}$P-ATP. Subsequent PCR reactions generated a single end-labeled probe from −146 to −73 of the hCRH proximal promoter. For the methylation interference assays, after PCR, the DNA was partially methylated using dimethyl sulfate as described (13). For uracil interference studies, dUTP was added at a concentration of 50 μM to the PCR reactions to generate probes containing partial substitution with deoxyuracil. For generation of completely deoxyuracil-substituted probes, 400 μM dUTP replaced TTP in the amplification reactions. Because JEG-3 nuclear extracts contain nuclease activities that degrade deoxyuracil-substituted DNA, nuclear extracts used for interference experiments were partially purified to remove this activity.

Binding reactions for interference assays contained 40,000 cpm of either partially methylated or partially deoxyuracil-substituted end-labeled DNA, and purified JEG-3 nuclear extract as described for EMSA. For each probe, five identical reactions were performed and loaded in adjacent lanes on gels for electrophoresis. After electrophoresis, gels were transferred to diethylaminoethyl-81 paper (Whatman, Maidstone, England) without fixation and exposed to storage screens (Molecular Dynamics, Sunnyvale, CA) for 2 h at −20 C. The locations of the shifted band and free probe for the five lanes were identified using a model 425B PhosphorImager (Molecular Dynamics), and excised in block, electroeluted, and precipitated. Eluted DNA was resuspended in PCR buffer (Life Technologies, Gaithersburg, MD) with 5 mM $MgCl_2$. Deoxyuracil containing DNA was cleaved using uracil DNA glycosylase (Life Technologies). Reactions contained 0.2 U uracil DNA glycosylase and were incubated at 37 C for 30 min, and then 30 min at 90 C. Partially methylated DNA was similarly cleaved by incubation in PCR buffer with 5 mM $MgCl_2$ for 30 min at 90 C. Formamide dyes were added, and equal counts were loaded onto sequencing gels containing 6 M urea, 8% 38:2 (acrylamide-bis), 0.5 × TBE [45 mM Tris-HCl (pH 8.3), 1.4 mM EDTA, 56 mM boric acid, final concentration]. After electrophoresis, fixation, and drying, the results were visualized using storage screens and by autoradiography at −80 C with intensifying screens.

### Partial Purification of JEG-3 Nuclear Extract

Crude JEG-3 nuclear extracts were prepared as previously described (10). All subsequent steps were performed at 4 C. Nuclear extracts were pooled, diluted 5-fold with column buffer [20 mM Tris-HCl (pH 7.8), 0.1 mM EDTA, 50 mM KCl, 0.1 mM DTT, 10% glycerol], and loaded onto a 5-ml heparin agarose type 1 H 6508 column (Sigma, St. Louis, MO) that had been equilibrated with the same buffer. The column was washed with 5 ml of buffer, and 1 ml fractions were eluted with a 20-ml linear gradient from 50 mM to 1 M KCl in the same buffer. Fractions were monitored using conductivity, and selected fractions were dialyzed for 4 h on ice into column buffer containing 50 mM KCl, and then dialyzed overnight on ice into 20 mM HEPES (pH 7.9), 50% vol/vol glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT. Individual fractions were stored at −20 C and assayed for binding activity using EMSA. Fractions were also assayed for the presence of nuclease activity by performing EMSA with the completely deoxyuracil-substituted probe and determining the preservation of intact free probe. Fractions with peak binding activity were also free of nuclease activity.

### UV Cross-Linking of Protein to DNA

UV cross-linking and analysis of resulting labeled proteins were performed using a standard protocol with minor modifications (13). The BrdU DNA probe, hCRH −146 to −73, was generated using PCR, by including 200 μM BrdU (Sigma) and $\alpha^{32}$P-dATP during the amplification. Each binding reaction combined 40,000 cpm of this labeled, BrdU-substituted DNA, with either 20 μg BeWo nuclear extract, 17.4 μg JEG-3 nuclear extract, or 9.4 μg Rcho-1 nuclear extract. Incubations were performed as described for EMSAs. When indicated, 10 pmol unlabeled competitor were added. After overnight incubation at 0 C, binding reactions were exposed for 5 min to UV light using a Fotodyne UV transilluminator (Hartland, WI). Each sample then received 2 μl of 50 mM $CaCl_2$, 10 U DNase I (Boehringer Mannheim), and 1 U micrococcal nuclease (Sigma). Digestion of DNA was 30 min at 37 C. Samples were precipitated with 25 μl 20% trichloroacetic acid, resuspended in loading buffer, boiled for 5 min, and then resolved by electrophoresis on Laemmli discontinuous 10% 38:2 (acrylamide-bis) polyacrylamide gels. Gels were fixed, transferred to 3MM chromatography paper (Whatman), and dried. The results were visualized using autoradiography at −80 C with an intensifying screen or by storage screen analysis. The mol wt of the human-specific activator was determined by comparison to $^{14}$C mol wt standards (Life Technologies) using ImageQuant 2.0 software (Molecular Dynamics).

### EMSA Supershift Assays

Antibody to ATF-2 (SC-187X) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody to CREB (catalog no. 9192) was the generous gift of Andreas Nelsbach (New England Biolabs, Beverly, MA). Oligonucleotides for generating a 41-bp duplex radioactive probe containing a CRE were dGATCGGATCCGATTGCCTGACGTCAGAGAGC and dCGATAGATCTGCTCTCTGACGTCAGGCA. These were annealed and 3′-end labeled using Klenow fragment. GEM ATF-2 plasmid DNA, a gift from John J. Keilty (University of Massachusetts, Worcester, MA), was used to program the T7 TNT reticulocyte lysate system (Promega) for *in vitro* translation of ATF-2. Nuclear extracts containing CREB were prepared from BE(2)-C cells and Hela cells as described above.

Binding reactions for the CREB probe were performed in 25 μl reactions containing 10 mM HEPES (pH 7.8), 1 mM spermidine, 3 mM $MgCl_2$, 7.2% glycerol, 0.6 mg/ml BSA, 0.06% Nonidet P-40, 3 mM DTT, and 150 μg dI·dC (38). When indicated, 1 pmol of unlabeled competitor DNA was included

in the reaction. Reactions contained 2 μl of lysate or nuclear extract and were incubated for 10 min at 20 C before addition of specific probe. After addition of probe DNA, incubation continued for an additional 10 min at 20 C. When indicated, 1 μl of antibody was added, and incubation for all reactions was continued for an additional 10 min at 20 C and then at 0 C overnight. Samples were resolved on 20 cm × 0.5 mm nondenaturing 4% 80:1 (acrylamide-bis) gels containing 2.5% glycerol, 0.5× TBE and electrophoresis was in 0.5× TBE buffer.

Supershift binding reactions for the CRH probe were performed in the usual fashion with the inclusion of antibody and an additional 10 min of incubation at 20 C before the incubation at 0 C overnight and resolution by nondenaturing gel electrophoresis.

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# Expression of Human CRF Transgenes in Transgenic Mice: Analysis of Species-Specific Placental Expression*

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Running Title: **hCRF Transgenic Mice**

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**Abstract:**

Transgenic mice were created carrying a transgene composed of 5 kb of the human corticotropin releasing factor (CRF) promoter linked to the coding region of neomycin phosphotransferase II. Evaluation of four independent lines of transgenic mice confirmed expression of the transgene in hypothalamus, and, in three lines, appropriate regulation in response to stress. The expression of the transgene in other organs, in both males and females, was similar to the expression pattern of the endogenous mouse CRF gene. Evaluation of transgenic placentas, however, showed no expression of the hCRF transgene in 21 of 22 specimens. Therefore, hCRF transgene expression in placenta is similar to the endogenous mouse CRF gene, rather than reflecting the human expression pattern.

These results indicate that the 5'-flanking control region dictates both hypothalamic expression and stress regulation of CRF expression. The lack of placental expression of the hCRF transgene supports our previous cell culture studies which indicated a dominant role for species-specific trans-acting factors in determining placental expression of the hCRF gene.

**Introduction:**

Corticotropin releasing factor, CRF, is a 41 amino acid amidated neuropeptide. Expression of CRF in the paraventricular nucleus of the hypothalamus plays a key role in regulating the hypothalamic-pituitary-adrenal axis, which is responsible for the synthesis of adrenal glucocorticoids. The regulation of hypothalamic CRF expression is complex. The normal circadian expression pattern of CRF is modulated by glucocorticoids via an inhibitory feedback pathway operating at the level of gene transcription (1,2). CRF is also induced by a variety of physiologic stresses and is a common feature of the stress response (3). In stress, CRF stimulates increased adrenal production of glucocorticoids; yet, in response to stress, the expression of CRF is not decreased by the elevated levels of circulating glucocorticoids (3). These features of CRF regulation, as well as the actual peptide sequence of CRF, are conserved across numerous animal species, emphasizing the integral role of CRF in maintaining mammalian homeostasis (3).

Expression of CRF is not restricted to the hypothalamus (3). A major site for peripheral expression of CRF is placenta, but its expression in placenta is species-specific (3,4). Humans and high primates express CRF in the placenta. Other species, including rat, mouse, guinea pig, and lemur do not (4,5). Furthermore, in humans, the pattern of placental CRF expression is different from that of hypothalamus. Unlike hypothalamic CRF, human placental CRF expression is neither circadian, nor negatively regulated by glucocorticoids (6).

Humans are the only species expressing high levels of CRF at term, a pattern not even observed in chimpanzees (7,8). Likewise, humans are the only species known to express the specific CRF binding protein, in maternal circulation (9). Both of these observations suggest a very unique and species-specific role for CRF in human physiology.

Our recent studies of placental CRF expression have exploited the availability of both human and rat choriocarcinoma cells as models for placental trophoblasts (10). These studies support a dominant role for transacting factors rather than DNA sequence differences in dictating the observed species-specific placental expression patterns (10). While the use of trophoblast cell lines is appropriate and has allowed a detailed molecular analysis of CRF expression, the determinants of placental CRF expression have not been evaluated in an *in vivo* system. Although CRF transgenic mice have been reported using rat CRF genes (11,12), no transgenic mice have been made using the human CRF gene in a manner suitable for these types of investigations of placental CRF expression. In this study we report analyses of transgenic mice carrying a human CRF transgene.

**Materials and Methods:**

**hCRF-Neo Transgenic Mice.**

The human CRF genomic clone, SpHCRH-1, was the generous gift of Shosaku Numa (13). The 5 kb upstream region was isolated as previously described (10) as a EcoRI-XhoI fragment using TthIII1 partial digestion, ligation to an XhoI linker, and digestion with EcoRI. This CRF promoter was ligated to sequences encoding neomycin phosphotransferase II (Neo) (14) and SV40 splice and polyadenylation signals derived from the plasmid RSV-Neo (15). The entire transgene containing the 5 kb CRF promoter, the 1 kb Neo coding sequence, and the 1.6 kb SV40 derived splice and polyadenylation sequences, was isolated as an EcoRI fragment. Injections were performed at DNX corporation (Princeton, N.J.). Tail DNA preparations were analyzed by transfer to nylon membranes and hybridization to radioactive DNA probes derived from the 1 kb BglII-SmaI fragment of the Neo gene. Four transgenic founder animals were obtained, and transgenic lines were established by breeding to non-transgenic B6/SJL mice. F1, F2 and subsequent breedings to obtain homozygous mice were performed, and offspring were analyzed to confirm inheritance of the CRF-Neo transgenes.

**Primers for Reverse transcription PCR.**

Mouse placental lactogen II (mPLII) primers were designed to cross intronic sequences and amplify a cDNA sequence of 432 bp. Primers were dACATCACGACACTTCAGGACC and dGACCTATGGCCTGATGTTAAGC.

Amplification was performed in 1.5 mM $MgCl_2$ for 40 cycles at an annealing temperature of 55° C. For amplification of the Neo gene, primers were designed to amplify a 450 bp sequence containing a NcoI restriction site. Primers were dTTGTCACTGAAGCGGGAAG and dCAAGCTCTTCAGCAATATCACG. Amplification was performed in 1.5 mM $MgCl_2$ for 40 cycles using an annealing temperature of 55° C.

**Stress Responses and Ribonuclease A/T1 Protection Assays.**

Homozygous adult female mice from each of the four CRF-Neo transgenic lines were analyzed to evaluate the responsiveness of the CRF-Neo transgene to stress. Animals from each line were either injected intraperitoneally with 40 mg/kg metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone, Sigma, St. Louis, MO) in dimethylsulfoxide in the late afternoon (stressed), or not treated (control). Stressed animals were kept at 4° C. overnight. Control animals were housed at ambient temperature. The following morning, the hypothalamic region was dissected to isolate the region of the paraventricular nucleus and total RNA was prepared for analysis as described (16).

The antisense Neo RNA was prepared from a pBKSII(-) Neo construct, containing the Neo gene (14) as a BglII - SmaI 1 kb fragment. The construct was linearized at the NcoI site and was used as template for T7 RNA polymerase which synthesized an antisense Neo RNA of 410 bp. The human gamma actin gene cloned in pGEM7Z, (a gift from C. Semenkovich, Washington University), was used as template for the actin control antisense

RNA using T7 RNA polymerase. RNA probes were labelled using $\alpha$-[$^{32}$P] CTP. Probes were then hybridized to 20 ug total RNA from the tissue and processed according to the manufacturer's specifications (RPA II Kit, Ambion Inc., Austin, TX). The reactions were then resolved by electrophoresis on 6% denaturing polyacrylamide gels, and exposed to storage screens overnight. The assays were visualized and quantified using a model 425 B PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant 2.0 software. End labelled DNA standards were used to confirm the size of the protected fragments. The autoradiogram image was processed for presentation using NIH Image 1.59.

**Evaluation of Organ and Placental Expression by Reverse Transcriptase PCR.** For evaluation of the expression of the transgene, organs were harvested for RNA preparation and analysis from homozygous male and female transgenic mice. For analysis of placental gene expression, males from three of the four transgenic lines were bred with non-transgenic females. Placentas and fetuses were isolated for analysis of RNA and DNA at early, mid, and late gestation (term). RT PCR analysis for Neo and mPLII expression was performed using minor modifications of published techniques (17). RNA was isolated using the protocol of Chomczynski and Sacchi (16,18) with an additional precipitation step using 5 M KOAc (pH 5). To remove any low level DNA contamination prior to reverse transcription, RNA samples were treated with RQ1 DNAse (Promega, Madison, WI) at 37° C. for 15 minutes, then at 65° C. for 20 minutes to inactivate the enzyme. After cooling, the specific antisense

primers were added to 2 ug RNA and heated at 70° C. for 10 minutes, then quick chilled on ice. MMLV polymerase reaction cocktail (United States Biochemicals, Cleveland, OH), without enzyme, was added, and samples were incubated at 37° C for 2 minutes. MMLV polymerase (United States Biochemicals, Cleveland, OH) was added and incubation continued for one hour at 37° C. The reactions were diluted 1:10 with UV treated 20 mM Tris-HCL (pH 7.4), 0.1 mM EDTA. For each PCR reaction, 5 ul of this reverse transcriptase product was added to a PCR reaction cocktail. For the intronless Neo gene prior to PCR, the PCR reaction mix (without Taq DNA polymerase and the RT product DNA) was incubated for one hour at 37° C. with NcoI followed by inactivation at 65° C. for 20 minutes. NcoI cuts the Neo gene between the two primers, and thus would prevent amplification of any contaminating DNA. All PCR solutions were made in UV treated water, and precautions were taken to prevent contamination of the PCR reactions with DNA containing Neo sequences. PCR reactions were for 40 cycles, using optimized $MgCl_2$ concentrations and cycling temperatures for each primer set. Samples were resolved on 2 % agarose gels in Tris-Borate-EDTA, stained with ethidium bromide, photographed, and then transferred to Magna nylon membranes ( MSI, Westboro, MA).

**Hybridization Analysis.**

Following a 30 hour transfer to nylon membrane, the DNA was UV crosslinked to the membrane at the maximum crosslinking setting of 1200 J. The membranes were hybridized overnight at 42° C. in a formamide hybridization cocktail containing the 1 kb BglII-SmaI Neo fragment labelled by random priming (Quick Prime Kit, Stratagene, La Jolla, CA). The next day the membranes were washed twice at room temperature for 15 minutes in 1X SSC / 0.1% SDS, followed by two washings at 42° C. for 15 minutes in 0.1X SSC / 0.1% SDS. The membranes were exposed to x-ray film or storage screens.

**Results:**

**Human CRF-Neo Transgene.**

A hCRF-Neo transgene was constructed using the 5 kb human CRF promoter linked to the coding region for the Neo gene, and containing splice and polyadenylation sites from SV40 (Fig 1). The identical promoter and splice-polyadenylation signal linked to firefly luciferase as a reporter has been evaluated in human and rodent choriocarcinoma cell lines and has shown species and cell type-specific expression in transient transfections (10). Transgenic mice were created using this human CRF transgene to evaluate the expression of the human promoter in mouse placentas.

**Transgenic Mice Incorporated the CRF-Transgene.**

Four founder mice were identified by analysis of tail DNA. These four founder animals were bred to homogeneity. Inheritance of the transgene followed Mendelian pattern for somatic incorporation at a single site for each founder line (data not shown). The expression of the Neo gene was not expected to produce an observable phenotype. Indeed, no abnormalities of development were observed, nor was there evidence of decreased viability or fetal wastage.

**Regulated Hypothalamic Expression of the CRF-Neo Transgene.**

One characteristic of appropriate targeting and expression of the CRF gene shared by both humans and mice is expression in the paraventricular nucleus and induced expression of CRF in response to stress. Appropriate

targeting and expression of the CRF-Neo transgene in hypothalamus was confirmed using RT PCR (not shown) and RNase protection assays (Fig. 2). All four founder lines showed detectable basal expression of the CRF-Neo transgene in hypothalamus. Each line represents an independent insertion event, and there are quantitative differences in the levels of transgene expression. Three of four lines showed increased expression of the transgene in response to stress (Fig. 2). These data indicate that the transgene contains information dictating appropriate targeting to the hypothalamus and the regulatory sequences necessary for a stress response. Three lines, 97, 105, and 109, were examined further for expression of the hCRF transgene outside the brain.

**Expression in other organs.**

To determine if the transgene demonstrated expression patterns previously described for the endogenous mouse CRF gene, RNA from organs of adult male and female mice was analyzed. To maximize the ability to detect even small amounts of Neo transgene expression, RNA was subjected to RT PCR amplification, followed by transfer to membranes and hybridization using a specific Neo probe (Fig. 3 and data not shown). There was detectable expression of the transgene in hypothalamus and, in line 97, in non-hypothalamic brain (not shown). There was also expression in adrenal, ovary, and uterus, but not liver, from the three lines of transgenic mice examined (Fig. 3 and data not shown). Expression of the transgene, therefore, had a similar

distribution pattern as the endogenous mouse CRF gene, detected using a similar method (5).

**Placental Expression of the hCRF Transgene.**

In order to analyze the expression of CRF in the placenta, matings were performed between non-transgenic B6/SJL females and transgenic males from three founder lines. Since placenta is a fetal tissue, it carries the same genotype as the fetus. All maternal tissues, including uterus, a site of CRF expression (Fig. 3), are non-transgenic and thus cannot contaminate the placental samples with CRF-Neo expressing tissues. Pregnant female mice from these matings were sacrificed prior to parturition (15-21 days fetal development) and the individual placentas and fetuses were isolated. Fetuses were examined to confirm inheritance of the CRF-Neo transgene. RNA was extracted from the placentas of confirmed positive transgenic fetuses and was analyzed for Neo transgene expression, again using RT PCR followed by hybridization analysis on membranes (Fig. 4 and data not shown).The analysis focused on placentas isolated near parturition because in the human placenta CRF expression is highest during the third trimester of pregnancy. In these three independent lines of mice, evaluation of a total of 22 placentas, the CRF-Neo transgene is not expressed in all but one placental sample (Fig. 4 and data not shown). We have no definite explanation for this one exception, but consider a rearrangement or contamination as possibilities. As a control, all placentas examined expressed the mPLII gene (data not shown), a marker gene present in rodent trophoblasts

from day 10 through term (19). Placentas isolated from the mice during earlier stages of pregnancy also fail to express the transgene (data not shown). Thus, these transgenic mice express the hCRF transgene, but with a rodent pattern, rather than that of humans.

**Discussion:**

Our previous studies have examined the expression of hCRF in human trophoblast cell lines, rodent CRF in a rodent trophoblast cell line, and each gene in the opposite cell type (10). This approach has served to identify the species-specific contributions of sequence differences as well as trans-acting factors. The results we obtained in cell culture systems and *in vitro* using nuclear extracts, have now been confirmed and extended *in vivo*, using a human CRF-Neo transgene in mice.

The transgenic analysis of human-specific placental expression of CRF could not be performed in previously existing CRF transgenic mice, since these transgenes were created using the rat CRF gene (11,12). Our findings here confirm that although the human CRF promoter is expressed in mouse hypothalamus and in other organs in a pattern similar to that of the endogenous mouse gene (5), the human gene is not expressed in rodent placenta. The results reported here are consistent with our cell culture analyses, which demonstrated a species-specific expression pattern for the CRF gene using the same 5 kb promoter, and even with a 532 bp promoter (10). In these earlier studies, our comparison of mouse and human CRF promoters also clearly demonstrated a requirement for species-specific trans-acting factors in determining the placental expression pattern of the CRF gene (10). The alternate mechanism, relying on differences in DNA sequences between human and rodent CRF promoters, but conserved placental transcription factors, would

predict hCRF transgene expression in the mice. Transgenic experiments can never formally exclude potential contributions of more distal promoter regions. However, placental expression based only on human-specific DNA sequences is not supported by these transgenic results.

Previous studies of the expression of CRF transgenes has focused on expression of CRF in the hypothalamus and other central nervous system sites (11,12). These previous studies have indicated that in addition to contributions of the 5'-flanking region and promoter, sequences within the coding region and the 3'-end of the gene may also be necessary for targeted central nervous system expression (11,12). While the focus of our current studies was an evaluation of the placental expression of CRF, our transgenic studies do also provide some new insight regarding hypothalamic regulation of CRF; namely, that the 5 kb hCRF promoter contains sequences dictating expression in the paraventricular nucleus of the hypothalamus, and that this expression is appropriately regulated in response to stress.

Our previous studies identified a potential repressor site located between -532 and -400 bp. Removal of this region results in expression of hCRF reporter constructs in the rat choriocarcinoma cell line (10). A candidate nuclear factor from the rat choriocarcinoma cell line has been identified which binds to this region (10). It is possible that this region of the promoter and the corresponding nuclear factor(s) act to exclude expression of the CRF transgene in mouse placenta. It is also possible that this repressor function is involved in

extinguishing expression of CRF in other tissues. While these hypotheses cannot be tested using the current transgene, hCRF transgenic mice could be created with the repressor function deleted or mutated to determine whether removal of the repressor's binding site would result in CRF transgene expression in mouse placenta and in other organs.

Another neuroendocrine gene exhibiting a species-specific placental expression pattern is the alpha-subunit of CG (20). Like CRF, $\alpha$-CG is expressed in the placentas of humans, higher primates, and, uniquely, in horses. Expression in other species is restricted to the pituitary (20). A similar transgenic analysis of $\alpha$-CG expression, using a human $\alpha$-CG promoter in mice, reported an expression pattern distinct from our studies of CRF. The human $\alpha$-CG transgene was expressed not only in mouse pituitary, but also in mouse placenta (21). In contrast to our results, the expression for $\alpha$-CG demonstrates the dominance of human specific DNA sequences rather than human specific placental trans-acting factors in determining placental $\alpha$-CG expression.

Our knowledge regarding the role of placental CRF and its specific binding protein (22) in the physiology of human fetal development and parturition is still developing. Other species, including the sub-human primates, regulate labor without placental CRF, or without the same pattern of placental CRF expression. Although CRF is produced by placental trophoblasts (23), one might still entertain the possibility that placental CRF expression is only an

oddity or peculiarity of human placenta. There is abundant evidence that, in fact, regulation of human labor is distinct from other species, and that CRF does have an important, and perhaps central, role in human physiology and labor (23). Receptors for CRF have been found in uterine myocytes and in placenta (8). In human placenta, amnion, chorion, and decidua, CRF has been shown to increase production of prostaglandins which are known mediators of labor (24). Although a direct, independent effect of CRF on uterine contractility has not been demonstrated, CRF does augment the contractile effects produced by both oxytocin (25) and prostaglandin F$\alpha$ (26). CRF may also play a role in regulating placental perfusion (27,28,29), an important consideration in both normal and abnormal pregnancies. A recent study supports the role of CRF as a "placental clock," timing the onset of human labor (30). CRF was elevated in women destined to deliver preterm, and depressed in women destined to deliver post-term (30). These differences in CRF levels were apparent by the end of the first trimester and persisted throughout the pregnancy (30). Furthermore, the study showed that CRF concentrations increased dramatically at term, with a coincident drop in the level of CRF binding protein (30). These two changes increase the availability of free CRF in maternal plasma.

In addition to the roles of placental CRF in uterine function and labor, placental CRF may also activate the fetal hypothalamic-pituitary-adrenal axis. There is an increase in cortisol in fetal plasma during the last 5 weeks of

pregnancy (6), coinciding with the increases in CRF. Glucocorticoids are required for appropriate development of several fetal organs (31) and may also serve as one of the signals necessary for the initiation of labor (6,32). An additional role for placental CRF may be to regulate production of ACTH in the placenta, rather than the fetal pituitary, and stimulate the fetal adrenal glands (6).

Placental CRF may participate in human fetal development and also may be a major regulator of normal and abnormal human labor. Further understanding of the regulation of CRF in response to cellular signals and by human-specific mechanisms may contribute to a better understanding of physiology of normal and pre-term labor and to the development of clinical applications.

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## FIGURE LEGENDS

**Fig. 1: The human CRF-Neo transgene.** The transgene was assembled using the human genomic CRF promoter sequence, the coding region of the Neo gene, and splice and polyadenylation signals derived from SV40. The construct contains 5 kb of the CRF promoter. The coding region of the Neo gene indicates the BglII and SmaI sites used for generation of specific probes. The entire transgene was prepared as an EcoRI fragment and used for injection to generate transgenic animals.

**Fig. 2: Stress induces hypothalamic CRF-Neo transgene expression.** RNase protection assays were performed on total RNA isolated from the hypothalami of female mice. Labelled antisense Neo RNA spanning the coding region was used as probe for quantitation of CRF-Neo transgene expression. Labelled antisense gamma-actin was used for detection of actin expression as control. Independent lines of the transgenic mice are designated as 97, 98, 105, and 109 based on numbers assigned to the original founder animals. **(A):** RNase protection assay from representative control and stressed transgenic mice from line 105. Neo and gamma actin indicate the specific protected bands resulting from CRF-Neo and actin mRNA. **(B):** Relative CRF transgene RNA expression in control and stressed animals. For each animal, relative expression of the CRF-Neo transgene was normalized using the expression of the actin gene as standard, and designating the mean basal expression in line 97 as 1.0. Each bar represents the mean ± S.E.M. from three animals.

**Figure 3. Analysis of human CRF transgene expression in mouse organs.** RNA was prepared from organs isolated from transgenic male and female mice. Specific RT PCR amplification of the Neo transgene message was followed by resolution on agarose gels and transfer to nylon membranes. Gene expression was detected by hybridization using a fragment of the Neo gene as radioactive probe. Results from line 105 are shown. The arrow indicates the RT PCR product from amplification of the expressed CRF-Neo transgene mRNA. Organs are labelled as: UTR, uterus; TST, testis; OVR, ovary; LVR, liver; KID, kidney; HRT, heart; BRN-HYPO, brain without hypothalamus; ADR, adrenal.

**Fig. 4. Analysis of human CRF transgene expression in mouse placenta.** Total RNA was prepared from placentas isolated near parturition (18 - 21 days fetal development). Specific RT PCR amplification of the Neo transgene message was followed by resolution on agarose gels and transfer to nylon membranes. Gene expression was detected by hybridization using a fragment of the Neo gene as radioactive probe. Results from line 105 are shown. The arrow indicates the RT PCR product from amplification of the expressed CRF-Neo transgene mRNA. Brain RNA serves as positive control for detection of transgene expression. UV treated water and liver RNA are negative controls. Lanes 1-7 are individual placental RNA samples. BRN, RNA from transgenic adult brain; LVR, RNA from transgenic adult liver; WTR, UV treated water.

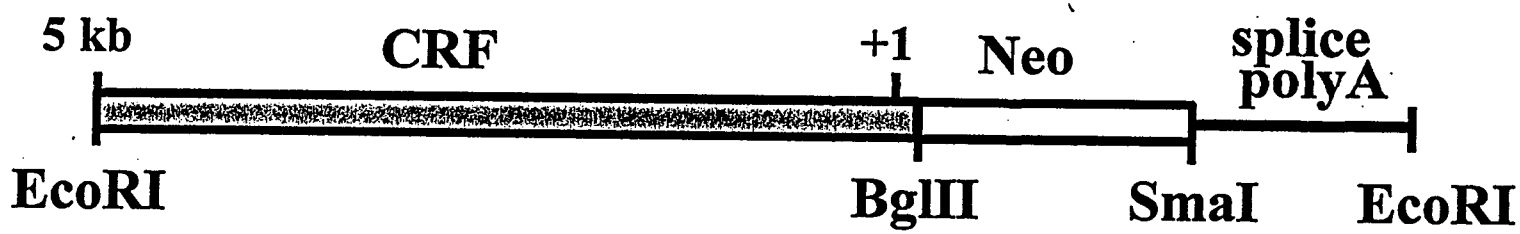


Figure 1

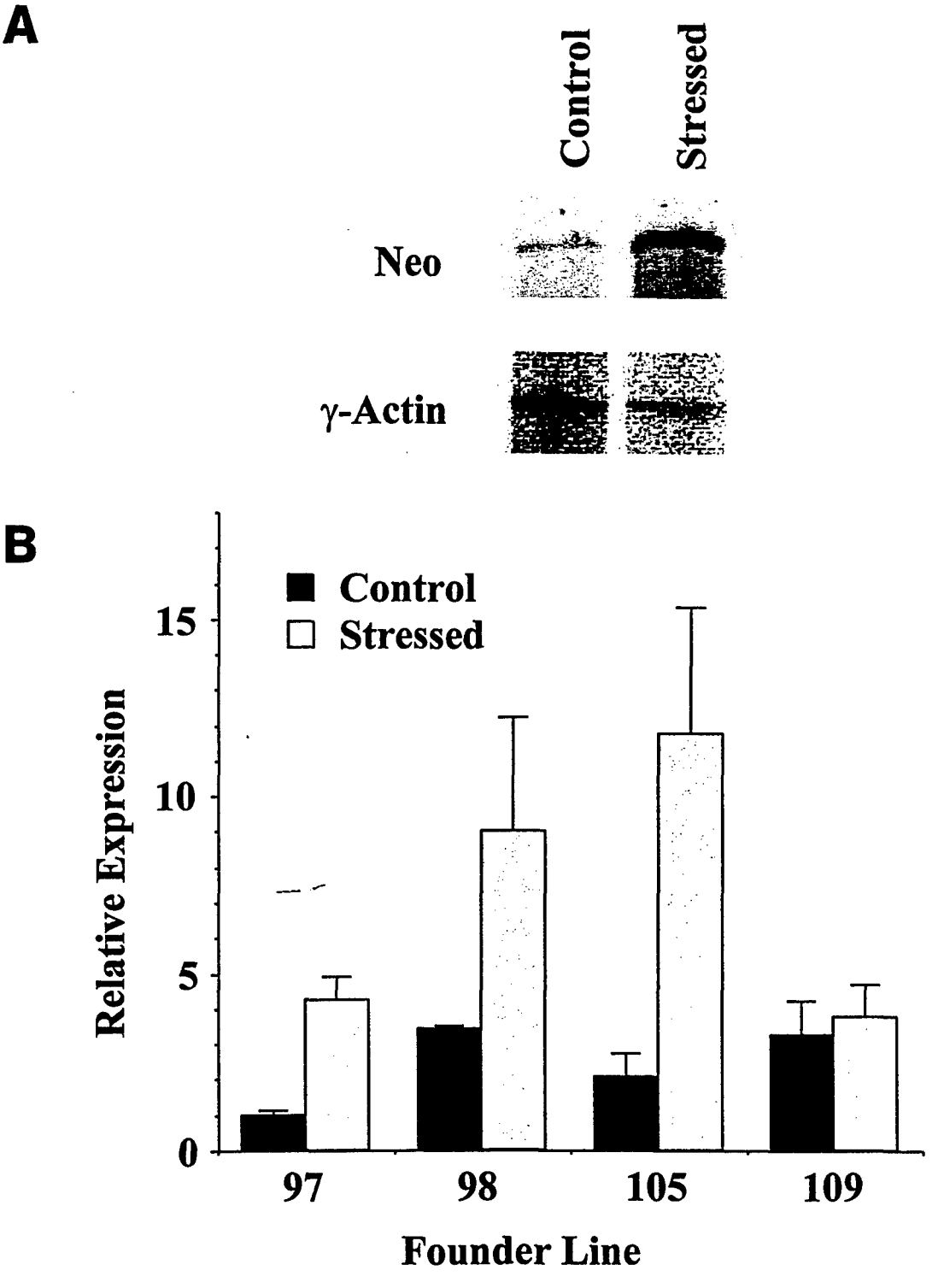
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Figure 2

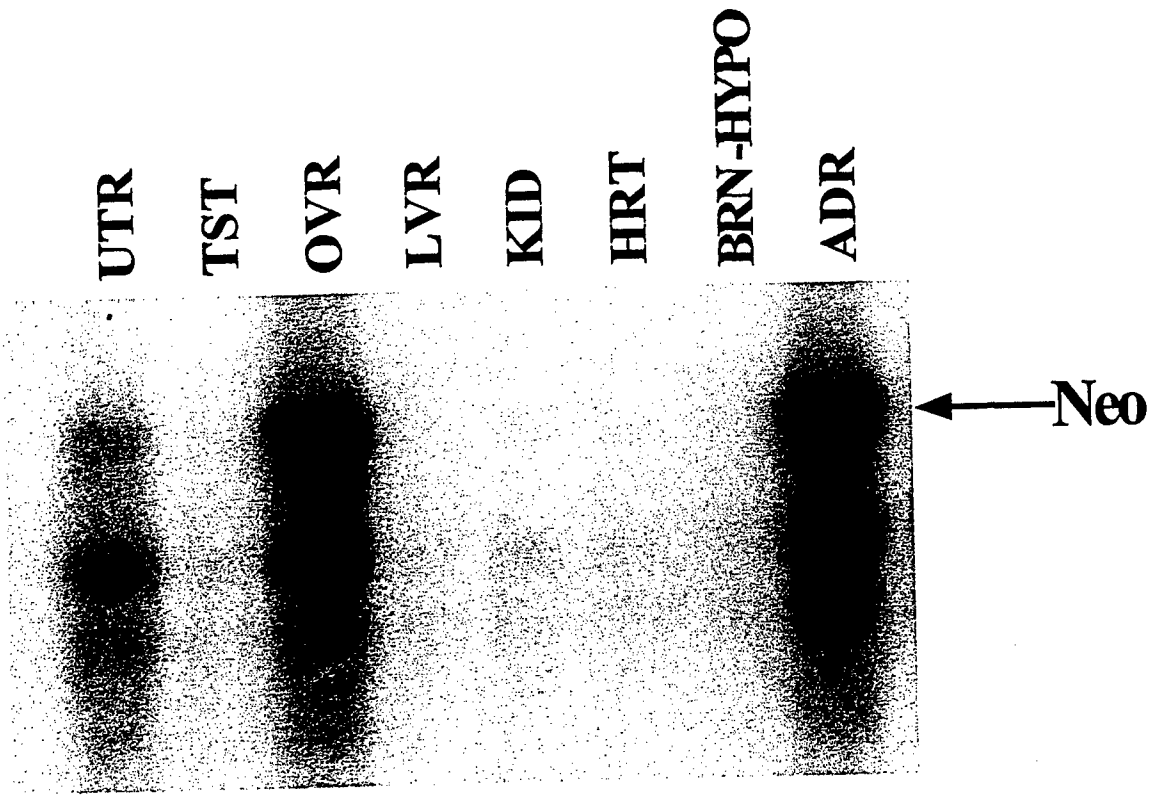


Figure 3

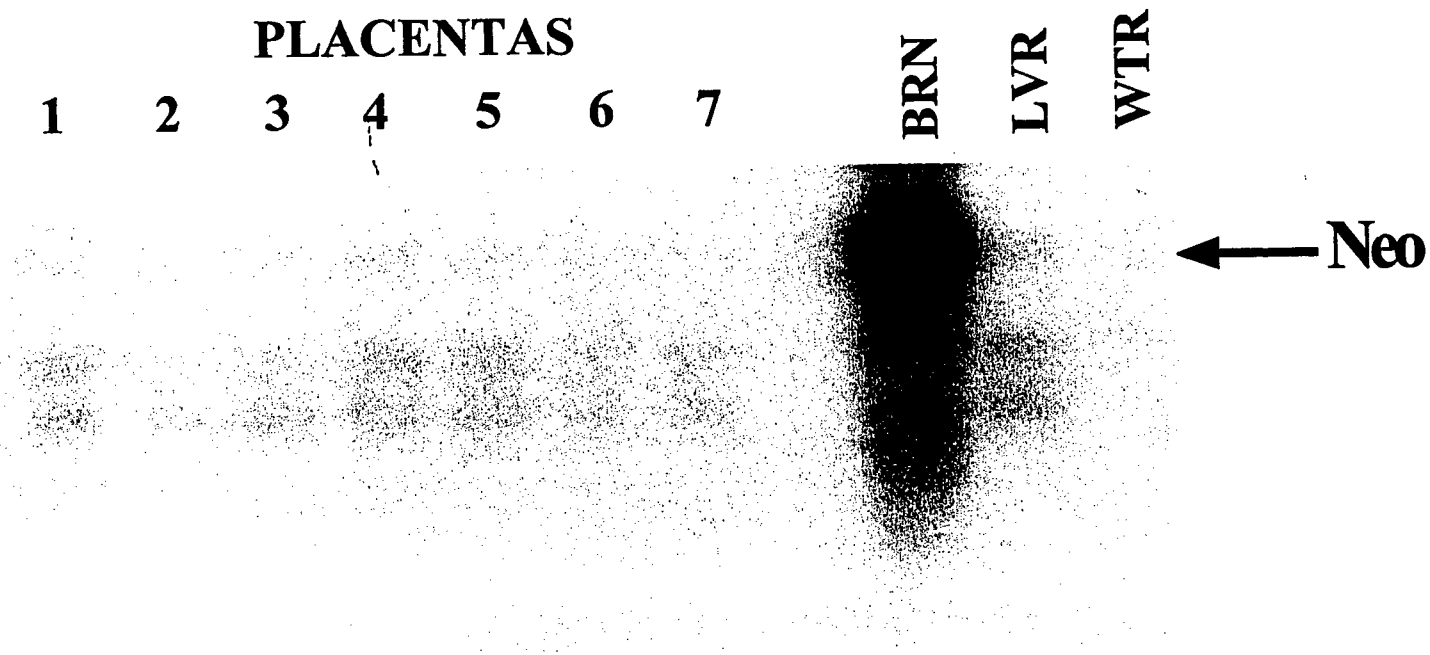


Figure 4

## Appendix.

### Distribution Statement.

All research summarized in this Final Report has been or will be submitted for publication in a peer-reviewed journal. In keeping with generally accepted principles and specific journal requirements, we have promised that as a pre-condition for publication, **no other publicity or distribution, including reports in the lay press, may precede the journal publication of our complete reports.** The data contained in this final report marked proprietary may be distributed on a confidential basis to enable appropriate review. Any other distribution should be postponed until after journal publication.

## Publications

**Scatena CD** and Adler S. 1996. *Trans*-Acting Factors Dictate the Species-Specific Placental Expression of Corticotropin-Releasing Factor Genes in Choriocarcinoma Cell Lines. *Endocrinology* 137: 3000-3008.

**Scatena CD** and Adler S. 1998. Characterization of a Human Specific Regulator of Placental Corticotropin Releasing Hormone. *Molecular Endocrinology* 12: 1228-1240.

**Scatena CD**, Ramkumar TP, and Adler S. Expression of Human CRF Transgenes in Transgenic Mice: Analysis of Species-Specific Placental Expression. Manuscript in Preparation.

## Presentations/Abstracts

**Scatena CD** and Adler S. 1994. Species-Specific Placental Expression of Corticotropin Releasing Hormone in Transgenic Mice and Choriocarcinoma Cell Lines. 76th Annual Meeting of The Endocrine Society. Anaheim, CA. The Endocrine Society Program and Abstracts, 1994: p. 568.

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